

**ROLE OF HUMAN PAPILLOMAVIRUS AND THE P53 GENE IN  
CUTANEOUS CARCINOGENESIS IN RENAL  
ALLOGRAFT RECIPIENTS**

**LESLEY ANN STARK**

**SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

**UNIVERSITY OF EDINBURGH**

**1994**



## **DECLARATION**

The work presented in this thesis has been carried out by myself except where specifically indicated in the acknowledgements and the thesis was composed by myself.

Lesley A Stark  
September, 1994



## **CONTENTS**

	<b>Page Number</b>
<b>Title Page</b>	1
<b>Declaration</b>	2
<b>Contents</b>	3
<b>Acknowledgements</b>	8
<b>Dedication</b>	9
<b>Abbreviations</b>	10
<b>Abstract</b>	11
<b>Chapter 1: Introduction</b>	13
<b>1.    Cutaneous Neoplasia In Renal Allograft Recipients</b>	14
1.1    Epidemiology and pathogenesis of cutaneous neoplasia in RARs	14
1.2    Aetiopathogenesis of cutaneous neoplasia in RARs	19
1.2.1    Host Factors	19
1.2.2    Environmental factors: Solar radiation	20
1.2.3    Environmental factors: Immunosuppression	21
1.2.4    Environmental factors: Viral infection	22
1.3    Molecular events associated with cutaneous carcinogenesis	24
1.3.1    Genetic abnormalities in cutaneous neoplasia	24
1.3.2    p53 and cutaneous carcinogenesis	24
1.3.3    Mouse model of skin carcinogenesis	25
1.3.4    Human skin carcinogenesis and RARs	27
<b>2.    Papillomaviruses And Cancer</b>	28
2.1    Virion properties and classification	28
2.2    Human papillomavirus infection	28
2.3    Papillomaviruses and cancer	30
2.3.1    Anogenital cancer	30
2.3.2    HPV, cutaneous cancer and epidermodysplasia verruciformis	32
2.3.3    HPV and cutaneous carcinogenesis in renal allograft recipients	32
2.4    Biology of papillomaviruses	33
2.4.1    Genome structure and function	33
2.5    Cellular transformation by HPV	38
2.5.1    Transformation by E6 and E7 from genital associated HPV types	39
2.5.2    Interaction of E6 and E7 from genital tract associated HPV types with cellular proteins	41

2.5.3	Cellular transformation by E6 and E7 of EV-associated HPV types	44
2.5.4	Genomic integration of HPV DNA	45
2.5.5	Additional events required for malignant progression	46
<b>3.</b>	<b>The p53 Tumour Suppressor Gene</b>	47
3.1	p53 and carcinogenesis	47
3.1.1	p53 mutations and 17p deletions in sporadic human tumours	47
3.1.2	Germline p53 mutations	47
3.1.3	Accumulation and mutation of p53 in cutaneous carcinogenesis	49
3.2	The gene for p53 and its transcript	49
3.3	Biological functions of wild type p53	51
3.3.1	p53 acts as a tumour suppressor	51
3.3.2	p53, DNA damage, apoptosis and the cell cycle	52
3.3.3	p53 and differentiation	55
3.4	Regulatory pathways of wild type p53	56
3.4.1	p53 is a transcriptional transactivator and repressor	56
3.4.2	p53 protein interacts with cellular proteins	58
3.5	Modification of wild type p53	58
3.5.1	Distribution and nature of p53 gene mutations in human tumours	59
3.5.2	Mutant p53 and transformation	60
3.5.3	Conformation and stability of wild type and mutant p53	60
3.5.4	Cellular proteins inactivate p53	62
3.5.5	Viral proteins inactivate wild type p53	62
3.6	Summary	66
3.7	Aims	68

<b>Chapter 2: Materials And Methods</b>	69
<b>1. Clinical And Histological Data</b>	70
1.1 Patient details	70
1.2 Tissue collection	70
1.3 Histopathological characterisation of lesions	71
<b>2. Preparation Of Purified HPV DNA</b>	72
2.1 Transformation of competent bacterial cells with HPV DNA	72
2.2 Small scale plasmid preparation	74
2.3 Large scale plasmid preparation	75
2.4 Validation of HPV plasmid DNA	76
2.5 Preparation of purified HPV DNA for use as probes	78

<b>3.</b>	<b>Southern Hybridisation Analysis</b>	79
3.1	Preparation of genomic DNA	79
3.2	Radioactive labelling of HPV DNA	80
3.3	Dot blot hybridisation of HPV plasmid DNA	80
3.3.1	Preparation of filters	80
3.3.2	Prehybridisation and hybridisation	81
3.3.3	Washing conditions	81
3.3.4	Re-probing	81
3.4	Southern hybridisation analysis of genomic DNA	82
3.4.1	Restriction digestion of genomic DNA	82
3.4.2	Southern transfer of genomic DNA	83
3.4.3	Prehybridisation and hybridisation	83
<b>4.</b>	<b>Polymerase Chain Reaction</b>	83
4.1	Primer design	83
4.2	PCR analysis	84
4.3	PCR analysis of genomic DNA	84
4.4	Consensus primer PCR	85
4.5	Procedures to minimise the risk of contamination	86
<b>5.</b>	<b>Immunocytochemistry</b>	86
5.1	Immunocytochemical assay for the detection of accumulated p53	86
5.2	Assessment of extent of immunopositivity	87
<b>6.</b>	<b>Single Strand Conformational Polymorphism And Sequencing To Detect p53 Mutations</b>	87
6.1	PCR of exons 5 to 8 of p53 for SSCP analysis	87
6.2	SSCP analysis of PCR products	88
6.3	Cloning of PCR products for sequencing exon 7 mutations	88
6.3.1	PCR of genomic DNA	88
6.3.2	Ligation	89
6.3.3	Sequencing	90
6.3.4	Polyacrylamide gel electrophoresis	90
6.4	Use of DYNABEADS for sequencing exon 5 mutations	91
6.4.1	PCR of genomic DNA	91
6.4.2	Preparation of single stranded DNA	91
6.4.3	Sequencing and electrophoresis	92
<b>Chapter 3:</b>	<b>Results</b>	93
<b>1.</b>	<b>Clinical And Pathological Features</b>	94

1.1	Clinical features	94
1.2	Histopathological features	95
<b>2.</b>	<b>HPV In Cutaneous Neoplasia Of RARs</b>	99
2.1	Design and optimisation of HPV detection assays	99
2.1.1	Validation of HPV probes	99
2.1.2	Optimisation of the Southern hybridisation assay	99
2.1.3	Design of PCR primers for HPV types 1,2,5 and 8	104
2.1.4	Optimisation of PCR assays for HPV types 1,2,5 and 8	104
2.2	HPV prevalence in cutaneous neoplasia from RARs and immunocompetent patients (ICPs)	105
2.3	HPV types and physical status of HPV DNA in cutaneous neoplasia from RARs	107
2.4	Prevalence of specific HPV types in cutaneous lesions from RARs and ICPs as determined by type specific PCR	114
2.5	Consensus primer PCR	118
2.6	Correlation of HPV DNA detection by Southern hybridisation and type specific PCR	121
2.7	Prevalence of HPV DNA in individual patients	123
<b>3.</b>	<b>p53 Accumulation And Mutation In Cutaneous Neoplasia From RARs</b>	126
3.1	Optimisation of immunocytochemical techniques	126
3.2	p53 accumulation in cutaneous lesions from RARs and ICPs	127
3.3	Distribution of accumulated p53	128
3.4	p53 immunoreactivity in cutaneous lesions from patients with differing susceptibility to neoplasia	130
3.5	p53 immunoreactivity and sun exposure	133
3.6	p53 mutations in cutaneous malignancies	133
3.7	SSCP analysis of p53 immunopositive and immunonegative lesions	136
<b>4.</b>	<b>p53 Abnormalities And The Presence Of HPV DNA</b>	137
4.1	HPV status and accumulated p53	137
4.2	HPV status and mutated p53	137
<b>Chapter 4:</b>	<b>Discussion</b>	139
<b>1.</b>	<b>Cutaneous Neoplasia In RARs</b>	140
1.1	Susceptibility to cutaneous neoplasia in RARs	140
<b>2.</b>	<b>HPV And Cutaneous Carcinogenesis Of RARs</b>	143
2.1	Prevalence of HPV DNA in spectrum of cutaneous neoplasia in RARs	143

2.2	Specific HPV types in cutaneous neoplasms from RARs	144
2.3	Physical status of HPV DNA in cutaneous lesions from RARs	146
2.4	Other HPV types in cutaneous neoplasms of RARs	148
<b>3.</b>	<b>p53 In Cutaneous Carcinogenesis Of Renal Allograft Recipients</b>	<b>150</b>
3.1	p53 accumulation and progression in cutaneous carcinogenesis	150
3.2	p53 accumulation in cutaneous neoplasia from RARs and ICPs	152
3.3	p53 mutation and cutaneous carcinogenesis	153
3.4	p53 accumulation and gene mutation in cutaneous carcinogenesis	155
<b>4.</b>	<b>Model Of Cutaneous Carcinogenesis In RARs</b>	<b>158</b>
4.1	The role of HPV in cutaneous carcinogenesis of RARs	158
4.2	The role of p53 in cutaneous carcinogenesis of RARs	160
4.3	The relationship between HPV and p53 in cutaneous carcinogenesis	161
4.4	The role of UV radiation in cutaneous carcinogenesis	163
4.5	A model of cutaneous carcinogenesis in RARs	164
4.6	Cutaneous carcinogenesis in individual patients	168
4.7	Final conclusions	169
<b>Chapter 5:</b>	<b>References</b>	<b>171</b>
<b>Appendix 1:</b>	<b>Relationship between RAR patient codes and sample numbers</b>	<b>210</b>
<b>Appendix 2:</b>	<b>Publications</b>	<b>216</b>

## **ACKNOWLEDGEMENTS**

I would like to acknowledge the following for their help with the work presented in this thesis: my supervisor, Professor CC Bird for his guidance and encouragement; Mark Arends for his useful discussions and ideas; Dr Benton for collecting samples and collating clinical data; Dr McLaren for histological assesement of specimens and help with the interpretation of immunocytochemical staining patterns; Jill Bubb and Robert Morris for answering my innumerable questions throughout the years of my Ph D; Joan Flannigan for help with DNA preparation and sample collection; John Lauder for instruction and help with immunocytochemistry; Gillian Stark and Tim Curtis for proof reading my thesis; Margaret Stark for help with the reference list and for giving up her new computer and printer for three months; and the Scottish Home and Health Department for providing me with funding.

I would also like to give a very special thankyou to my mum and dad who helped and supported me in every way possible during the time this thesis took to produce.

## **DEDICATION**

This thesis is dedicated to my boyfriend Allan and my son Josh.

## **ABBREVIATIONS**

BCC	Basal cell carcinoma
BPV	Bovine papillomavirus
DDW	Deionised, distilled water
DNA	Deoxyribose nucleic acid
EDTA	ethylene diamine tetra-acetate
EV	Epidermodysplasia verruciformis
HPV	Human papillomavirus
ICP	Immunocompetent patient
IEC	Intraepidermal carcinoma
K	Keratosi
Kb	Kilobase
KDa	Kilodalton
mRNA	messenger RNA
OD	Optical density
ORF	Open reading frame
PV	Papillomavirus
RAR	Renal allograft recipient
RNA	ribonucleic acid
rpm	revolutions per minute
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
SSC	Standard sodium citrate
TBE	Tris borate EDTA
TE	Tris EDTA
URR	Upstream regulatory region
US	Uninvolved, sun exposed, forearm skin
UV	Ultra violet
VW	Viral wart



## **ABSTRACT**

It is well established that renal allograft recipients have an increased incidence of viral warts and premalignant and malignant cutaneous lesions, and the risk of their development increases in proportion to duration of graft survival. It has been postulated that, in addition to the effects of prolonged immunosuppression and previous sun exposure, human papillomaviruses may also contribute to the carcinogenic process. In this study, the prevalence of human papillomavirus DNA was examined in a range of premalignant and malignant cutaneous tumours from 52 immunosuppressed patients (49 renal allograft recipients plus 3 cardiac allograft recipients) and 83 immunocompetent patients using Southern hybridisation analysis as a low stringency screening method and type specific polymerase chain reaction (PCR) assays for 8 human papillomavirus types. The combined results for renal allograft recipients showed that human papillomavirus was detectable in 71% of viral warts, 43% of premalignant keratoses, 42% of intraepidermal carcinomas, 51% of invasive squamous cell carcinomas and 14% of uninvolved skin specimens. The prevalence of human papillomavirus DNA was significantly greater in squamous cell carcinomas ( $p < 0.02$   $\chi^2$  test) and intraepidermal carcinomas ( $p = 0.05$   $\chi^2$  test) than in uninvolved skin from renal allograft recipients. In immunocompetent patients, the pattern of human papillomavirus prevalence was 100% for viral warts, 15% for keratoses, 20% for intraepidermal carcinomas, 27% for squamous cell carcinomas and 8% for uninvolved skin. No single human papillomavirus type predominated in tumour specimens from either group. More tumours were found to contain human papillomavirus DNA by Southern hybridisation analysis than by type specific PCR indicating the presence of human papillomavirus types other than 1, 2, 5, 6, 8, 11, 16 and 18 in some tumours. This was confirmed in a small number of lesions found to be human papillomavirus positive by Southern Hybridisation analysis that were further typed by restriction digestion analysis. However, "low risk" human papillomavirus types 1, 2 and 6 as well as "high cancer risk" types 5 and 16 were specifically detected by PCR in a small number of neoplasms. These data suggest that multiple human papillomavirus types may contribute to cutaneous neoplasia in renal allograft recipients and that they appear to act early in the carcinogenic process, possibly by functioning as tumour promoters via stimulation of cell proliferation.

This study also employed immunocytochemical techniques to investigate the prevalence and localisation of accumulated p53 in over 200 cutaneous biopsies (including 56 squamous cell carcinomas) from renal allograft recipients and

immunocompetent controls. In renal allograft recipients, accumulated p53 was present in 24% of uninvolved skin samples, 14% of viral warts, 41% of pre-malignant keratoses, 65% of intraepidermal carcinomas, and 56% of squamous cell carcinomas (intraepidermal carcinomas differed significantly from keratoses ( $p<0.05$ ) and squamous cell carcinoma and intraepidermal carcinoma differed significantly from uninvolved skin ( $p<0.005$ ) and viral warts ( $p<0.01$ )). A similar trend was revealed in immunocompetent patients (an older, chronically sun-exposed population) but with lower prevalence of p53 immunoreactivity: 25% of uninvolved skin samples, 0% of viral warts, 25% of keratoses, 53% of intraepidermal carcinomas and 53% of squamous cell carcinomas. These differences were not statistically significant. Morphologically, p53 immunoreactivity strongly associated with areas of epidermal dysplasia and the abundance of staining correlated positively with the severity of dysplasia. These data suggest that p53 plays a critical role in skin carcinogenesis and is associated with progression to the pre-invasive state. However, the prevalence of accumulated p53 was similar in intraepidermal carcinomas and squamous cell carcinomas indicating that other genetic events must occur prior to the development of the invasive state. Single strand conformational polymorphism analysis (exons 5 to 8) was used to determine the frequency of mutated p53 in 28 malignancies with varying degrees of immunopositivity. p53 mutations were found in 5/9 (56%) malignancies with p53 staining in  $>50\%$  of cells reducing to 1/6 (17%) where 10-50% of cells were positively stained and none where  $<10\%$  of cells were stained. These data imply that factors other than p53 gene mutation play a part in the accumulation of p53 in skin cancers. No correlation was observed between stabilised p53 and the presence of human papillomavirus DNA in any of the lesions and p53 mutations were detected in human papillomavirus positive (including human papillomavirus 5 and 16) and human papillomavirus negative cancers. On the basis of the findings of this study, a model of cutaneous carcinogenesis in renal allograft recipients has been postulated.

**CHAPTER1:**  
**INTRODUCTION**

## **CHAPTER 1: INTRODUCTION**

### **1: CUTANEOUS NEOPLASIA IN RENAL ALLOGRAFT RECIPIENTS**

It is now well established that immunosuppressed allograft recipients are at an increased risk of developing certain types of cancer (Sheil et al, 1985; Alloub et al, 1989; Barrett et al, 1993). Kaposi's sarcoma, non-Hodgkin's lymphoma and carcinoma of the cervix, vulva and perineum, are especially prevalent. However, the most common cancer occurring in renal allograft recipients (RARs) is squamous cell carcinoma (SCC) of the skin (Hoxtell et al, 1977; Blohme & Larko, 1984; Sheil et al, 1985; Shuttleworth et al, 1987; McLelland et al, 1988; Hartevelt et al, 1990). Viral warts (VWs) and SCC precursor lesions are also more prevalent in transplant patients and form a continuous spectrum of cutaneous disease. Ultraviolet (UV) radiation and immunosuppression are known to be important in the aetiology of SCCs in RARs and there is evidence to suggest that human papillomaviruses (HPVs) may also play a significant role. However, the biological mechanisms by which these aetiological agents contribute to the neoplastic process of RARs, and their associated genetic events, have yet to be elucidated.

#### **1.1 EPIDEMIOLOGY AND PATHOGENESIS OF CUTANEOUS NEOPLASIA IN RARS**

The development of SCCs in RARs is a serious clinical problem and is proving to be one of the major long term complications of renal transplantation. The incidence increases logarithmically with the duration of immunosuppression and is highest in Caucasian transplant patients living in tropical or subtropical climates. In a large series reported from Southern Australia, 25% of RARs surviving 9.9 years and 50% of those surviving 20 years developed SCCs (Dyall-Smith et al, 1991), whereas in South East Scotland Barr et al (1989) found 2% of RARs with a graft life of 5 years or less and 13% of patients with a graft life of 5 to 22 years were affected by cutaneous SCCs. In

one other study from the Netherlands, the overall risk of developing a first skin cancer increased from 10% after 10 years to 40% after 20 years of graft survival (Hartevelt et al, 1990). Squamous cell carcinomas from RARs tend to be more aggressive, have a higher potential for local recurrence and metastasise more frequently than those from age-matched, immunocompetent controls (Hoxtell et al, 1977; Benton et al, 1992; Blohme & Larko, 1984; Hartevelt et al, 1990; Sheil et al, 1985). In some series, 13% of SCC from RARs have been reported to metastasise and deaths from cutaneous SCCs were 10 times higher than expected in these patients (Rowe et al, 1992). Some RARs exhibit multiple skin cancers which may recur soon after surgery, presumably because of the widespread dysplastic change which is evident in the sun-exposed skin of these patients (Figure 1.1A) (Benton et al, 1992). Atypical histological features, such as an overall verrucous architecture, hyperkeratosis and perinuclear halo formation, which are usually associated with viral infection, are often observed in SCCs from RARs indicating a possible viral contribution (Figure 1.1B) (Blessing et al, 1989). Basal cell carcinomas (BCCs) are also observed in RARs, however, the increase in SCCs with immunosuppression is significantly greater than BCCs. Indeed, the ratio of BCCs to SCCs is reversed in RARs from the 3:1 to 7:1 seen in the immunocompetent population to 1:1.2 to 1:3 in immunosuppressed patients (Rudlinger et al, 1986; Barr et al, 1989; Hartevelt et al, 1990; Benton et al, 1992).

Squamous cell carcinomas are commonly preceded by the development of VWs in RARs. In contrast to such lesions from immunocompetent patients (ICPs), VWs may be extensive, intractable and cause severe morbidity in allograft recipients (Figure 1.2A) (Rudlinger et al, 1986; Rudlinger & Grob, 1989; Bunney et al, 1992; Benton et al, 1992). As with skin cancer, they are more prevalent in patients with high sun exposure and usually occur on sun exposed body sites (Boyle et al, 1984; Shuttleworth et al, 1987). Although the incidence increases with the duration of immunosuppression (eg. Barr et al, (1989) reported a 77% incidence of VWs in patients with graft survival

of 5 to 22 years), VWs may appear just months after transplantation and are a recognised risk factor for skin cancer development (McLelland et al, 1988; Barr et al, 1989; Blessing et al, 1989). Many of the warts observed in RARs have a typical clinical appearance. However, those arising on sun exposed body sites are often atypical and may be difficult to distinguish from pre-malignant keratoses or SCCs on clinical grounds alone (Bunney et al, 1992; Ledo, 1992). These VWs may also show unusual histopathological features including epithelial dysplasia and the typical papilliferous architecture associated with HPV infection (Figure 1.1B and C) (Blessing et al, 1989; Barr et al, 1989).

Actinic keratoses, which are the most common precursor lesions to intraepidermal carcinomas (IECs) and SCCs, are also more prevalent in RARs and are often widespread, involving both sun exposed and covered areas (Blohme & Larko, 1984; Shuttleworth et al, 1987; McLelland et al, 1988; Marks et al, 1988 and 1992; Dodson et al, 1991; Kwa et al, 1992; Bouwes Bavinck et al, 1993a). It is in such areas of widespread keratoses that SCCs frequently arise (Figure 1.1A). Histologically, actinic keratoses from RARs often have a papillomatous (viral) architecture and show more severe epidermal dysplasia (sometimes approximating that seen in IEC) as compared to similar lesions from ICPs (Figure 1.1D). (Blessing et al, 1989; Hardie et al, 1980).

Intraepidermal carcinomas (Bowen's disease) have a long latency period in ICPs and only a small proportion of patients go on to develop frankly invasive SCC (Braverman, 1991; Johnson et al, 1992; Kwa et al, 1992). By contrast, IECs from RARs are much more aggressive and undergo accelerated progression to SCC (Blohme & Larko, 1984; McLelland et al, 1988; Shuttleworth et al, 1987; Bouwes Bavinck et al, 1993a).

Anogenital cancer is also a major clinical problem and a more frequent cause of death than skin cancer in transplant patients (Rudlinger et al, 1986; Halpert et al, 1986;

**Figure 1.1.** (A) Severe actinic damage with warts, keratoses and several foci of squamous cell carcinoma on the scalp of a 45-year-old man who had received a renal transplant 20 years previously. (B) Invasive squamous cell carcinoma (right) showing an overall verrucous architecture and hyperkeratosis, in continuity with typical fronds of a viral wart (left). (C) High power (x40) of B showing perinuclear halo-formation (which is a typical cytological feature of viral infection) along side severely dysplastic cells (D) Extensive keratoses and warts arising in a background of sun damaged skin on the dorsum of the hand of a male allograft recipient. Although many of these lesions showed dysplasia, none were malignant.

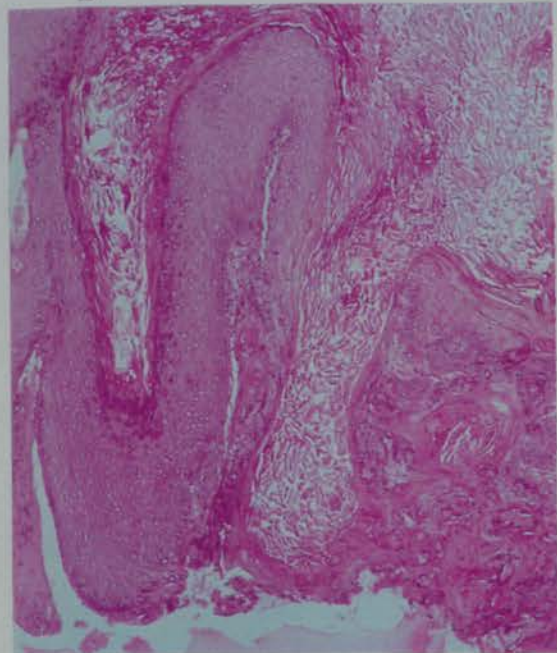


**FIGURE 1.1:- CLINICAL AND HISTOLOGICAL FEATURES OF CUTANEOUS LESIONS**  
**FROM RENAL ALLOGRAFT RECIPIENTS**

**A**



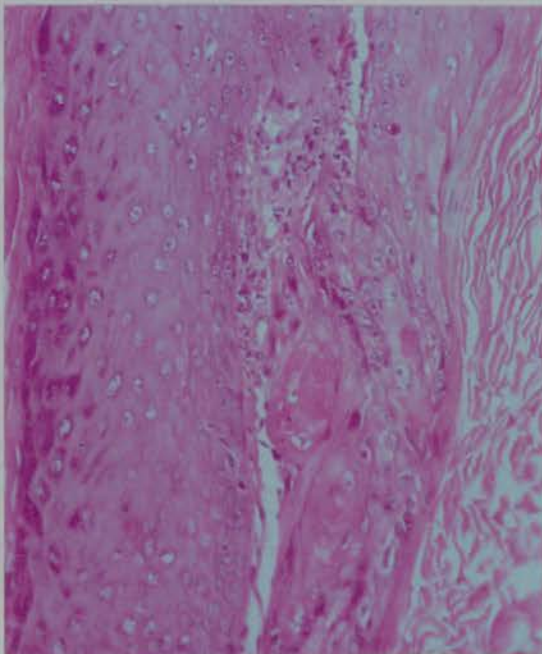
**B**



**D**



**C**





Alloub et al, 1989). In one study from the South East of Scotland, a significantly increased prevalence of cervical intraepithelial neoplasia was found in RARs (49%) compared with a control group of patients (10%) (Alloub et al, 1989). The detection of HPV 16 and 18 was also more common in RARs. Some of the long standing allograft recipients included in Alloub's series developed invasive SCC of the cervix, vulva and anal canal, as well as intraepithelial neoplasia and anogenital warts.

## **1.2 :- AETIOPATHOGENESIS OF CUTANEOUS NEOPLASIA IN RARS**

Both host and environmental factors have been implicated in the development of skin cancer in RARs. Age, skin type and HLA class I and II antigens are all considered to influence host susceptibility to skin cancer while solar radiation, immunosuppressive therapy and viral infection are believed to be the most important environmental risk factors.

### **1.2.1 Host Factors**

The high incidence of skin neoplasia among fair skinned RARs, especially those of Irish, Scottish and English descent, suggests that host skin pigmentation strongly influences susceptibility to the development of SCCs and VWs in RARs (McLelland et al, 1988; Dyall-Smith et al, 1991; Bouwes Bavinck et al, 1993a). A correlation between age and incidence of skin neoplasia has also been demonstrated (Blohme & Larko, 1984). However, this is probably more strongly linked to the number of years on immunosuppressive therapy and the cumulative level of UV exposure.

The major histocompatibility complex (MHC) contains a large number of polymorphic genes that encode the class I (HLA-A,B,C) and II (HLA-Dr,Dq,Dp) antigens which play an important role in the cellular immune response to viral and tumour antigens (Browning & Bodmer, 1992). HLA associated susceptibility exists for several kinds of viral related malignancies including Kaposi's sarcoma (HLA-Dr5), Burkitt's lymphoma

(HLA-A1,-B12 and -Dr7) and cervical carcinoma (HLA-Dqw3) (Jones et al, 1980; Scorza et al, 1986; Wank & Thomssen, 1991). A negative association between HLA-A11 and carcinoma of the cervix has also been reported (Wank & Thomssen, 1991). It has recently been demonstrated that HLA-A11 has a protective effect against skin cancer in RARs whereas HLA-Dr7, HLA-B27 and HLA-Dqw2 are associated with an increased risk of developing SCCs (Bouwes Bavinck et al, 1991a and b and 1993b; Glover et al, 1993). Furthermore, HLA-DR homozygosity and HLA-B mismatching are significantly associated with an increased risk of SCC in RARs (Bouwes Bavinck et al, 1991a). These data indicate that there may be an inherited susceptibility to skin cancer development involving genes in the class I and II regions of MHC. Recent studies have also demonstrated that susceptibility to the immunosuppressive effects of UVB radiation may be genetically determined (Yoshikawa et al, 1990).

### **1.2.2 Environmental Factors: Solar Radiation**

The association between human skin cancer and sun exposure has been recognised since the 19th century. The preferential localisation of cutaneous neoplasia on sun exposed skin and the increase in incidence nearer the equator, strongly suggest an important role for sunlight in the pathogenesis of skin disorders in RARs (Boyle et al, 1984; Dyll-Smith et al, 1991; Shuttleworth et al, 1987; Hartevelt et al, 1990). Indeed, numerous studies have demonstrated a highly significant association between sun exposure and skin cancer in both RARs and ICPs (Vitalliano & Urbach, 1980; Marks, 1992; Johnson et al, 1992; Kwa et al, 1992). Cumulative lifetime exposure to UV radiation has been shown to be important in the development of skin cancers, whereas the development of keratotic skin lesions (including VWs) is thought to be connected with recent episodes of sun burn (Bouwes Bavinck et al, 1993a). Solar radiation may enhance the development of cutaneous neoplasia by two distinct mechanisms: as a direct carcinogen and as an immunosuppressant (Kripke, 1984; Brash et al, 1991; Yoshikawa et al, 1990; Streilein, 1991). Although UVB light (290

to 320 nm) is the most carcinogenic wavelength, its effects are augmented by UVA light (320 to 400 nm) which acts as a co-carcinogen. The carcinogenic effects of UV radiation result directly from the absorption of UV light by DNA. The UV induced photoproducts (principally cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts) are potentially mutagenic if located within oncogenes or tumour suppressor genes (McGregor et al, 1991; Brash et al, 1991).

Epidermal Langerhans cells play a critical role in initiating the local immune response against virally infected cells and skin cancers (Streilein, 1991; Baadsgaard, 1991; Cruz & Bergstresser, 1989). Low doses of UVB have been shown to deplete mouse epidermis of the surface markers on Langerhans cells and reduce the total number of these cells (Aberer et al, 1981; Stingl et al, 1981). Most murine tumours induced by UV radiation are highly immunogenic and are rejected when transplanted into normal syngeneic mice. However, when such tumours are transplanted into UV irradiated mice, the tumours grow progressively (Fisher & Kripke, 1977). This unresponsiveness is due to the appearance of antigen specific suppressor T lymphocytes (and thus systemic immunosuppression) following high doses of UV radiation (Ullrich & Kripke, 1984). Urocanic acid is a major UV absorbing component of the stratum corneum which undergoes a *trans* to *cis* isomerization on absorption of UV. The induction of *cis*-urocanic acid is also associated with systemic immunosuppression of UV irradiated mice (Noonan & De Fabo, 1992). Although most of these studies have been carried out on experimental animals, more recent data suggests that UV radiation can alter immune function in humans in a similar fashion (Baadsgaard, 1991).

### **1.2.3 Environmental Factors: Immunosuppression**

Epidermodysplasia verruciformis (EV) is a rare inherited skin disorder which is associated with specific defects of the cell mediated immune response and the life-long presence of multiple, persistent VWs (Fuchs & Pfister, 1990). In addition, 30%-50%

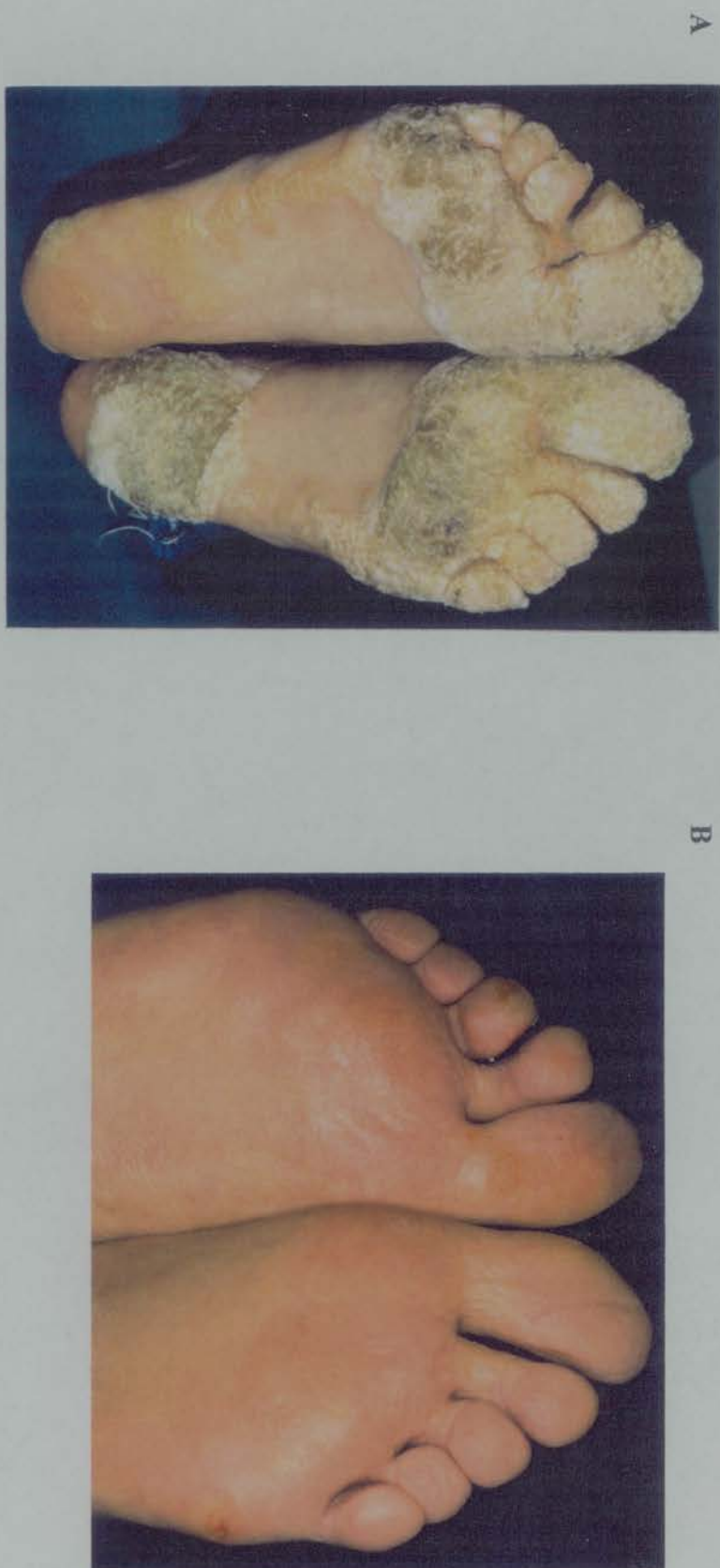
of EV patients develop cutaneous SCCs (Orth et al, 1979; Pfister et al, 1983a; Orth, 1986). Viral warts and skin cancers are also more prevalent in patients with other genetically determined conditions associated with defects of the immune system (Briggaman & Wheeler, 1979; Barnett et al, 1983). Similarly, HPV infection is a common occurrence in therapeutically immunosuppressed, non-transplant patients (Benton et al, 1992). These data provide circumstantial but compelling evidence that immunosuppression plays a major role in the neoplastic process of RARs. Further evidence that immunosuppression is contributing to the development of at least VWs in RARs comes from the observation that extensive VWs may resolve rapidly when graft rejection occurs and immunosuppressive drugs are withdrawn (Figure 1.2A and B) (Benton et al, 1992). It is presently unknown whether a similar reversal of the malignant process occurs following graft rejection, although, limited data suggests that the development of cutaneous cancers may at least slow down (Benton et al, 1992). The increased incidence of both VWs and skin cancers in RARs and other immunosuppressed patients suggests that immunosuppression may contribute to cutaneous carcinogenesis by allowing persistence of viral infection.

#### **1.2.4 Environmental Factors: Viral Infection**

Renal allograft recipients have a marked increase in malignancies that may be associated with viral infection such as carcinoma of the cervix (HPV), B-cell lymphoma (Epstein Barr virus (EBV)), and Kaposi's sarcoma (EBV) (Birkeland, 1983; Sheil et al, 1985; Alloub et al, 1989; Penn, 1991; Barrett et al, 1993). Although there is a strong association between the number of warts and the occurrence of SCCs in RARs (skin cancers having the histological appearance of a viral associated lesion) it has not yet been clarified whether viral infection is also contributing to the pathogenesis of skin malignancies in RARs. HPV will be discussed in detail in Section 2 of the Introduction.



FIGURE 1.2:- REGRESSION OF EXTENSIVE VIRAL WARTS IN A RAR FOLLOWING GRAFT REJECTION



**Figure 1.2 (A)** Extensive plantar mosaic warts in a renal allograft recipient of long standing. **(B)** Plantar warts fully regressed after graft rejection and removal of immunosuppressive drugs.

### **1.3 MOLECULAR EVENTS ASSOCIATED WITH CUTANEOUS CARCINOGENESIS**

#### **1.3.1 Genetic Abnormalities In Cutaneous Neoplasia**

The *ras* gene family is made up of three closely related genes (*Ha-ras*, *Ki-ras* and *N-ras*) which encode membrane bound proteins with intrinsic GTPase activity. Transforming *ras* mutations, mainly in codons 12, 13 and 61, appear to be common events in a large variety of human tumours. Although the majority of reports suggest that *ras* gene mutations are rare events in skin carcinogenesis (Campbell et al, 1993c; Mukhtar & Bickers, 1993), *Ha-ras* codon 12 mutations have been demonstrated in 46% of cutaneous SCCs in one series (Pierceall et al, 1991b).

DNA aneuploidy has been found in 25% to 80% of cutaneous SCCs (Frentz & Moller, 1983; Newton et al, 1987; Randall et al, 1990). Actinic keratoses and IECs may also be aneuploid but they demonstrate intralesional variation in chromosome number (or DNA index) suggesting origin from multiple cell clones with varying DNA content (Frentz & Moller, 1983). In contrast, SCC cell populations demonstrate little or no variation in aneuploid number/DNA index which supports origin from one cell clone (Frentz & Moller, 1983; Newton et al, 1987). Consequently, it has been proposed that selection and dominance of a single cell clone in epidermal pre-cancers is associated with the emergence of tumour invasion (Kwa et al, 1992).

#### **1.3.2 p53 And Cutaneous Carcinogenesis**

One of the putative major biological roles for p53 is to prevent replication of genomes that have suffered DNA damage through induction of growth arrest and/or apoptosis in response to cell injury (Lane et al, 1992 and 1993). It therefore seems likely that p53 may play an important role in the skin where epidermal keratinocytes are continually exposed to the damaging effects of UV radiation. Although p53 mutations have been extensively investigated in a wide range of human cancers, the role of p53 in cutaneous carcinogenesis of RARs and ICPs has yet to be elucidated. The biological and

biochemical properties of p53 and its role in cancer development will be discussed in Section 3 of the introduction.

### **1.3.3 Mouse Model Of Skin Carcinogenesis**

Information regarding the cellular and genetic mechanisms involved in the initiation and progression of human cutaneous neoplasia is presently limited. However, a number of the genetic events associated with different stages of the mouse 2-step model of cutaneous carcinogenesis have been characterised. This model provides a useful comparison in the interpretation of data derived from investigations into human skin cancer.

Initial treatment of mouse skin with a subcarcinogenic dose of a carcinogen such as dimethylebenzanthracene (DMBA), induces a population of initiated cells. Subsequent treatment with a non-carcinogenic promoting agents (such as phorbol esters) selectively expands the population of initiated cells and benign papillomas develop within three months. A proportion of these lesions spontaneously progress to malignant carcinomas. Therefore, this system possesses at least three well defined stages: initiation, promotion and progression (Yuspa et al, 1986 and 1991; Bowden & Krieg, 1991; Burns et al, 1991).

Initiation occurs rapidly, is irreversible and is commonly caused by mutagens. Although initiated cells persist throughout the lifetime of the animal, they do not develop into tumours without further treatment. Point mutation and/or amplification of the c-Ha-*ras* oncogene has been demonstrated in more than 90% of DMBA initiated mouse tumours (Quintanilla et al, 1986). This mutation is found in both papillomas and carcinomas suggesting that c-Ha-*ras* activation occurs early in tumour development (Balmain et al, 1984; Harper et al, 1987; Burns et al, 1991).

Tumour promotion in mouse skin is a complex process in which many of the biochemical changes are reversible (Hennings et al, 1984). Several applications of tumour promoter are usually required before papillomas form and in some cases, papillomas will regress when the promoter is withdrawn (dependent papillomas) (Hennings et al, 1984; Yuspa et al, 1985). The phorbol ester class of tumour promoters were traditionally used in mouse skin carcinogenesis protocols and are therefore the most extensively characterised (Scribner et al, 1983; Hennings & Yuspa, 1985; Fujiki et al, 1991). Phorbol esters activate protein kinase C and have the ability to stimulate the proliferation of some epidermal cells and induce differentiation of others (Hartley et al, 1985 and 1987). The net result of this heterogeneous response to promoting agents is the expansion of the proliferative population of cells at the expense of the differentiating population with each promoter exposure. Initiated cells are resistant to differentiation induced by phorbol esters and are therefore at a growth advantage (Kulesz-Martin et al, 1983; Hennings et al, 1984). Based on this data, it has been proposed that phorbol ester-mediated promotion involves changes in the growth and maturation kinetics of the skin, which leads to selective clonal expansion of initiated cells and papilloma formation.

Malignant conversion is a relatively rare event that occurs late in the mouse model of carcinogenesis (Bowden & Kreig, 1991). *In vivo*, it is often a variable process that is preceded by progressive dysplastic changes. The introduction of certain oncogenes (such as *neu*) into papilloma cell lines accentuate the dysplastic epidermal changes in the resultant tumours *in vivo* but these tumours do not convert to malignancy (Dotto et al, 1988). Several lines of evidence suggest that a single genetic change is sufficient to cause malignant conversion in mouse skin carcinogenesis. Activating mutations in the proto-oncogene *c-fos* and the tumour suppressor gene *p53* have been closely linked with this stage (Greenhalgh et al, 1990; Kemp et al, 1993).



#### **1.3.4 Human Skin Carcinogenesis And RARs**

The spectrum of cutaneous neoplasia observed in RARs, which progresses from benign VWs through increasing severities of dysplasia to invasive SCCs, makes this an ideal model in which to investigate the role of viruses, oncogenes and tumour suppressor genes at different stages of human skin carcinogenesis. Investigation of the putative roles of HPV and p53 in cutaneous carcinogenesis of RARs forms the basis of this thesis and these will be considered in more detail in the subsequent sections.

## **2: PAPILOMAVIRUSES AND CANCER**

### **2.1 VIRION PROPERTIES AND CLASSIFICATION**

Papillomaviruses (PVs) are a genus of DNA viruses that specifically infect squamous epithelia, inducing cellular proliferation and subsequently tumours of the skin or mucosa. Although these tumours, called papillomas or warts, are often benign, certain members of the PV family are associated with the development of malignant tumours (Arends et al, 1990; Chang, 1990; Quan & May, 1991; Howley, 1991) .

Papillomavirus virions are made up of a central core of closed circular, double stranded DNA of approximately 8 Kb, encapsulated in an outer protein shell of 72 capsomeres. Unlike some other DNA viruses they have no outer envelope. They are classified according to the host species they infect and their degree of nucleic acid homology with known PV types, as measured by hybridisation in the liquid phase (de Villiers, 1989). For a PV to be recognised as an independent type, the nucleotide sequence of its E6, E7 and L1 open reading frames (ORFs) must share less than 90% sequence homology to sequences of other PV types (Van Ranst et al, 1994). Over 70 independent HPV types have been identified to date which can be grouped according to the site of their associated lesions (Table 1.1).

### **2.2 HUMAN PAPILOMAVIRUS INFECTION**

Direct access to the basal layer of differentiating epithelium is thought to be necessary for a productive HPV infection to be established (Schneider, 1993). This occurs most commonly through microlacerations or by direct contact with basal cells at the transformation zone of the cervical squamo-columnar junction. The productive life cycle of the virus is highly dependent on the environment provided by differentiating keratinocytes and there is evidence that HPV can modify this keratinocyte maturation

**TABLE 1.1:- HUMAN PAPILLOMAVIRUS TYPES GROUPED ACCORDING TO THE SITE OF THEIR ASSOCIATED LESIONS**

Site	Group	HPV Type
Skin	A I	1
	A II	2, 3, 10, 27-29
	A III	4
	A IV	7
	A V	26
EV	B I	5*, 8*, 9, 12, 14#, 17#, 19, 20#-25, 36-38, 46, 47, 49
	B II	50
Mucosa And Specialised Skin	C I	6, 11, 13, 44
	C II	16*, 31#
	C III	18*, 32, 42, 45
	C IV	30#
	C V	33#
	C VI	34
	C VII	35#
	C VIII	39#
	C IX	41
	C X	43

Classification of the first 50 HPV types (except HPV 40 and 48) by site of infection and overall DNA sequence homology (Arends et al, 1990). The sites of viral infection are A = skin; B = skin of epidermodysplasia verruciformis (EV) patients; C = various mucosa and specialised skin (cervix, vulva, anogenital region, upper respiratory tract and digestive tract). \* = HPV types strongly associated with malignant tumours; # = HPV types suggested to have malignant potential or cloned from a cancer.

(Bedell et al, 1991). Early viral genes are expressed in the lower epithelial layers and regulate viral DNA synthesis, whereas the expression of capsid proteins and packaging of viral genomes is limited to the most highly differentiated upper layers (Sterling et al, 1990; Bedell et al, 1991; Meyers et al, 1992). Infectious viral particles may be released through expression of the viral E4 protein which has been shown to destabilise the intracellular cytokeratin network (Doorbar et al, 1991). Understanding of the pathogenesis of PV infection has been limited by the inability to propagate these

viruses in tissue culture. However, a collagen raft culture system containing cells derived from an HPV31b infected high grade epithelial dysplasia, has recently allowed the production of HPV 31b viral particles in the upper epithelial layers (Meyers et al, 1992). HPV 11 and 16 viral particles have also been produced using mouse xenograft systems (Kreider et al, 1987; Sterling et al, 1990). More data on the synthesis and assembly of virions and viral interactions with differentiating keratinocytes can be expected from studies using these and other experimental systems.

### **2.3 PAPILLOMAVIRUSES AND CANCER**

The role of PV in the aetiology of epithelial neoplasias was first demonstrated in early studies of the cottontail rabbit PV (CRPV) where 25% of CRPV-induced skin tumours in domestic rabbits progressed to malignancy (Lancaster & Olson, 1982). The first association of HPV with a form of human cancer was reported in skin cancers from patients with EV (Jablonska et al, 1972). More recently, the causative role of HPV in the induction of anogenital cancers has been documented and it is at this site that most information relating to the oncogenic potential of HPV has been generated (Arends et al, 1990; Chang, 1990; Quan & May, 1991; Howley, 1991; Zur Hausen, 1989 and 1991).

#### **2.3.1 Anogenital Cancer**

Many epidemiological surveys have demonstrated that early age at first coitus, multiple sexual partners and poor sexual hygiene are risk factors for the development of cervical cancer, implying that a sexually transmitted agent is a causal factor in the aetiology of this malignancy (Schneider, 1993; Brinton, 1992). It has also been demonstrated that wives of men with penile cancer have elevated rates of cervical cancer providing further epidemiological evidence that an infectious agent may be involved (Smith et al, 1980). The observation that cervical cancer often occurs in association with condylomata acuminata (genital warts), which are known to be caused

by HPV, provides further circumstantial evidence that HPV may play an aetiological role (Brinton et al, 1990; Palefsky et al, 1990). The first definitive evidence associating HPV with cervical cancer was the observation that the morphological abnormalities constituting cervical dysplasia are frequently associated with koilocytosis, which is the cytopathic manifestation of an HPV infection (Meisels & Fortin, 1976). Since these initial studies, numerous HPV prevalence studies have demonstrated HPV DNA in high proportions of cervical intraepithelial neoplasia (CIN) and squamous cervical cancers (Pater et al, 1986; Arends et al, 1991 and 1993; Zur Hausen, 1991; Lorincz et al, 1992).

It may be argued that HPV have a predilection for infecting CIN and are passengers rather than effectors in the carcinogenic process. Evidence to the contrary is provided by the fact that different HPV types are associated with specific anogenital lesions. Over 25 anogenital HPV types have so far been identified (Table 1.1) and these can be classified as either "low risk" or "high risk" based on the malignant potential of their associated genital tract lesions. The low risk types 6 and 11 are commonly associated with benign genital warts and low grade dysplasia (CIN 1) whilst the high risk HPV types 16 and 18 have been detected in association with up to 80% of high grade dysplasias (CIN 2 and 3) and up to 90% of invasive carcinomas (Pater et al, 1986; Arends et al, 1991 and 1993; Zur Hausen, 1991; Lorincz et al, 1992). Furthermore, there is an increase in the prevalence of the high risk HPV types and a corresponding decrease in the prevalence of the low risk HPV types with progression through the spectrum of condyloma, CIN (with increasing degrees of dysplasia), *in situ* and invasive carcinoma. Therefore, there is strong evidence in support of a causal role for certain types of HPV in the development of anogenital cancer.

### **2.3.2 HPV, Cutaneous Cancer And Epidermodysplasia Verruciformis**

Over 20 of the 70 recognised HPV types have been found in cutaneous neoplasms from patients with EV (Table 1.1). These patients suffer from extensive, debilitating VWs, pityriasis versicolor-like lesions, flat-topped wart-like plaques and *in situ* and invasive SCCs (Ostrow et al, 1982; Orth, 1986; Yabe et al, 1989; Fuchs & Pfister, 1990). A large range of HPV types have been isolated from the VWs of these patients but two specific types, HPV 5 and 8, have been found in up to 90% of SCCs (Orth et al, 1979; Pfister et al, 1981 and 1983a). Malignant transformation of VWs has also been observed in 25-30% of cases and HPV 5 DNA has been detected in metastatic SCC from EV patients (Ostrow et al, 1982; Yabe et al, 1989). These data provide strong evidence that high risk, skin-associated HPV types play a major role in cutaneous carcinogenesis in EV patients.

### **2.3.3 HPV And Cutaneous Carcinogenesis In Renal Allograft Recipients**

Clinical and histological evidence suggest that HPV may be a contributory factor in cutaneous carcinogenesis of RARs (see Section 1.1 of the Introduction). Furthermore, similarities exist between RARs and EV patients in that both have an increased incidence of VWs and skin cancers on sun exposed sites, and both have a depressed cell mediated immune system. However, investigations into the prevalence of HPV in skin malignancies of RARs have been inconclusive, with EV associated types and a variety of common cutaneous and genital HPV types being identified in some but not all studies (Lutzner et al, 1980 and 1983; Rudlinger et al, 1986; Van der Leest et al, 1987; Barr et al, 1989; Rudlinger & Grob, 1989; Dyll-Smith et al, 1991; Soler et al, 1992; Euvrard et al, 1993). Furthermore, the potential role of the virus in the cutaneous neoplastic process of RARs is presently incompletely understood.



## **2.4 BIOLOGY OF PAPILLOMAVIRUSES**

A causal role for HPV in the aetiology of anogenital cancer and cutaneous cancer from EV patients, as implied by epidemiological and prevalence studies, is supported by investigations into the molecular biology of the virus. These studies also provide information on the mechanisms by which HPV may contribute to the carcinogenic process.

### **2.4.1 Genome Structure And Function**

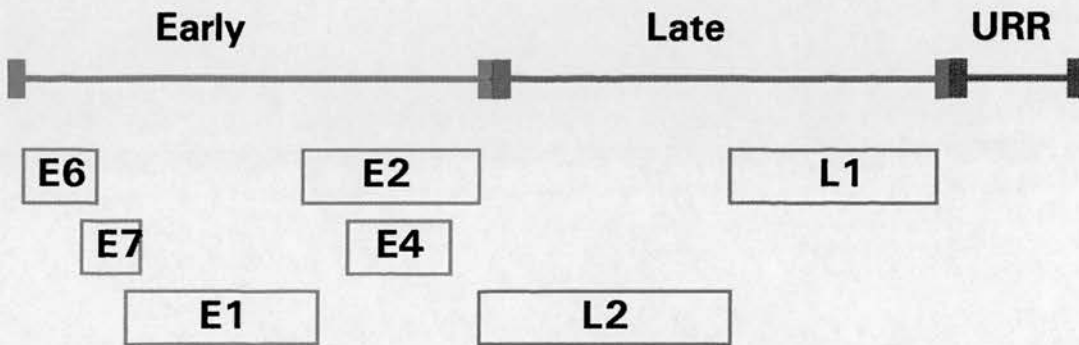
Sequence analysis of a number of PV genomes has revealed that all PV types exhibit a similar overall organisation (Baker, 1987; Ward et al, 1989; Sousa et al, 1990). The genome is divided into a number of ORFs which are all contained on a single coding strand of DNA (Figure 1.3). The other DNA strand contains only small, unconserved ORFs that are likely to be non-coding (Heilman et al, 1980). The early (E) region contains eight ORFs which code for proteins associated with genome replication and control. The late (L) region contains two large ORFs coding for the structural proteins of the viral capsid. The upstream regulatory region (URR) lies between the stop codon of L1 and the start codon of E6 and contains the promotor and enhancer elements required for regulation of viral transcription and replication. Functions were initially assigned to the ORFs using BPV1 as a model system. However, information on the transcriptional and functional organisation of HPV has more recently become available.

#### **L1 and L2**

L1 and L2 ORFs code for the viral structural proteins and are only expressed in terminally differentiated keratinocytes (Baker et al, 1987; Matlashewski, 1989; Sousa et al, 1990). The L1 ORF is the most highly conserved viral gene and codes for the major capsid protein which has an average molecular mass of 56KDa (Hjorth & Moreno-Lopez, 1982). This protein carries the major antigenic determinants for group specific cross reactivity within different groups of PVs (Nakai, 1987). L2 codes for a

protein of similar size but is one of the most poorly conserved viral proteins (Danos et al, 1983). Sequence analysis indicates that the L2 proteins fall into 3 homologous

**FIGURE 1.3:- GENOMIC STRUCTURE OF HPV 8**



The open reading frames (ORFs) of the coding strand of HPV8. Early (E) region ORFs code for proteins associated with viral replication and control of viral gene expression. Bovine papillomavirus and some anogenital HPV types have an additional E5 ORF, however, no ORF equivalent to E5 has been identified in EV-associated HPV types. The late (L) region contains two large open reading frames coding for the structural proteins of the viral capsid. The upstream regulatory region (URR) contains the promoter and enhancer elements required for regulation of viral transcription and replication.

group: fibropapilloma producing (BPV-1), cutaneous (cottontail rabbit PV (CRPV), HPV1a) and mucosal (HPV 6b, 11, 16 and 18) (Baker, 1987). Antigenic determinants carried by L2 do not cross react with antisera from any other PV, even within the same group of homology (Komly et al, 1986).

### E1 and E2

The full length E1 ORF is highly conserved among PV and encodes a 68KDa phosphoprotein with ATP dependent, helicase activity (Giri & Danos, 1986; Sousa et al, 1990; Lambert, 1991; Thorner et al, 1993). The E1 protein is essential for BPV1



extrachromosomal DNA replication and deletion or mutation results in viral integration into the host cell genome (Spalholz et al, 1993a and b). In addition to its positive replication function, the E1 protein of BPV1 is also capable of inducing transcriptional repression through the formation of a tight complex with the E2 transactivator protein (Sandler et al, 1993). E1 mRNA has been identified in cervical biopsies containing non-integrated HPV 16 DNA which suggests that the E1 protein may also be involved in replication of episomal DNA in HPV (Shirasawa et al, 1988). However, a 70KDa E1 protein has been identified in Hela cells which contain integrated HPV 18 DNA (Seedorf et al, 1987), implying that the E1 protein may have additional functions in HPV.

The transcriptional activation and repression of viral genes by different versions of the E2 gene product comprises one of the most important regulatory circuits of PV gene expression and appears to be a property shared by all PV types (Cripe et al, 1987; Chin et al, 1988; Ward et al, 1989; McBride et al, 1991; Lambert, 1991; Spalholz et al, 1993b). The BPV1 model has been used most extensively to characterise the products of the E2 ORF (Sousa et al, 1990; Lambert, 1991). E2 from BPV1 encodes 3 site specific, DNA binding proteins involved in transcriptional regulation of the viral genome (Monini et al, 1993; Lambert et al, 1989b)). The full length BPV1 E2 encodes a 48KDa protein which functions as a transcriptional activator (E2-Ta) (Spalholz et al, 1985; Androphy et al, 1987; Haugen et al, 1987; Lambert et al, 1989b). Two other polypeptides also encoded by the BPV1 E2 ORF are truncated forms of the E2 protein lacking the N-terminal transactivation domain. One of these is a 31KDa protein termed E2-Tr which specifically represses trans-activation by the full length E2 and is the most abundant of the 3 proteins (Lambert et al, 1987 and 1989b; McBride et al, 1988; Hubbert et al, 1988).

Less is known about the HPV E2 polypeptides. All the HPVs so far sequenced encode a full length E2 polypeptide analagous to the BPV1 transactivating protein (McBride et al, 1991). However, in contrast to BPV1, the major promoters of HPV 16 and 18 are repressed by the full length E2 protein (Thierry & Yaniv, 1987; Bernard et al, 1989; Romanczuk et al, 1990a and b). It is thought that the binding of E2 to two E2 binding sites adjacent to the TATA box of the promoters interferes with the assembly of the transcription complex (Romanczuk et al, 1990a). Disruption of the E2 ORF during viral integration has been implicated as a mechanism for HPV 16 or 18 induced carcinogenesis (see Section 2.5.4 of the Introduction) (Lazo et al, 1992; Krajinovic et al, 1993). The E2-Tr repressor protein or other truncated versions of E2 have not yet been identified for HPV. However, the C-terminal region of both the HPV 16 and HPV 11 E2 ORFs have the coding capacity for a repressor function similar to BPV-1 E2-Tr (Cripe et al, 1987; Chin et al, 1988).

## E4

The role of the E4 protein in the PV life cycle is still unclear. The RNA message formed from a single splice between the beginning of the E1 and E4 ORFs codes for the 17 KDa E1<sup>E4</sup> fusion protein which is the most abundant protein in HPV induced lesions (Breitburd et al, 1987; Zur Hausen, 1991; Steele et al, 1993). Up to 8 E4 protein species have been isolated from HPV 1 induced warts which appear to be specific for different layers of the epidermis and are thought to represent proteolysis products of the major E1<sup>E4</sup> protein (Breiturd et al, 1987). A similar complexity of E4 protein species has also been identified in HPV 2, HPV 4 and BPV 1 induced lesions and HPV 11 induced experimental tumours. The localisation and abundance of E4 in the more differentiated layers of the epithelia suggests that this is a late protein and may have a role in virion maturation. An association between HPV 16 E4 and the collapse of the cytokeratin network has recently been demonstrated suggesting that E4 may aid virion release through disruption of the cellular cytokeratin matrix (Doorbar et

may aid virion release through disruption of the cellular cyokeratin matrix (Doorbar et al, 1991). It has also been suggested that regulation of E4 expression throughout viral infection could modulate epithelial differentiation to favour different stages of the PV life cycle (Roberts et al, 1993).

## E5

The E5 ORF of BPV1 is responsible for 95-99% of the BPV-1 *in vitro* transforming activity (Yang et al, 1985; Campo et al, 1989; Horwitz et al, 1989; Sousa et al, 1990). This gene encodes a membrane bound, hydrophobic 7KDa protein which is found within cells primarily as a 15KDa homodimer (Schlegel et al, 1987). All transformation competent E5 mutants retain the ability to associate with the cellular membrane and to form dimers, revealing the importance of these functions in transformation (Spalholz, 1993). BPV1 E5 protein is also known to complex with both the 16KDa component of the vacuolar ATPase and the activated platelet derived growth factor receptor as a trimeric complex (Goldstein et al, 1991 and 1992).

The role of E5 in HPV induced lesions has yet to be elucidated. The E5 proteins from HPV types 6 and 11 share several structural similarities with BPV1 E5 and HPV types 6 and 16 E5 proteins exhibit transforming activity when assayed on NIH 3T3 and C127 cells (Chen & Mounts, 1990; Leechanachai et al, 1992). A recent study demonstrated that HPV 6 and 16 E5 proteins, like BPV 1 E5, are localised to cellular membranes and bind the 16KDa pore-forming protein component of vacuolar ATPase (Conrad et al, 1993). No ORF with homology to E5 has been identified in the EV-associated HPV types 5 and 8 (Fuchs & Pfister, 1990; Kiyono et al, 1992).

## Upstream Regulatory Region (URR)

The length of the URR varies between PV species but it has several conserved regions and maintains a similar overall organisation (Ward et al, 1989). The upstream region is

of the virus in infected cells, whereas the downstream end contains several promotor elements (TATA and CAAT boxes) for polymerase II binding and many repeats and inverted repeats commonly found in viral control regions (Waldeck et al, 1984; Howley, 1991). Also within the 5' region is the E2 enhancer/repressor which consists of at least 2 tandem repeats of the E2 binding motif (Lambert & Spalholz, 1987).

EV-associated HPV types seem to have a subgenus-specific organisation of the URR (Krubke et al, 1987; Ensser & Pfister, 1990). Sequence analysis of the URR of HPV types 5, 8, 19 and 25 has revealed that it is surprisingly short with a number of conserved features unique to EV-associated HPV types. There are four palindromic E2 binding sites, one binding site for the transcription factor jun/AP1, a series of more than 25 AT dimers and a number of additional motifs that are highly conserved among HPV 8 related HPV types (Ensser & Pfister, 1990). A number of features in the URR have been identified which separate different EV-associated HPV types into three groups. These groups do not correlate with the oncogenic activity of the HPV types, as measured by their detection in SCCs of EV patients.

## **2.5 CELLULAR TRANSFORMATION BY HPV**

Analysis of the biological functions of viral genes expressed in human tumours and manipulation of these genes in animal and human cell lines, has been used to support a role for HPV in cancer. These investigations have established E6 and E7 as oncogenes, found that high risk and low risk HPV types differ in their *in vitro* transforming activity and determined mechanisms by which E6 and E7 from the high risk genital HPV types may function.

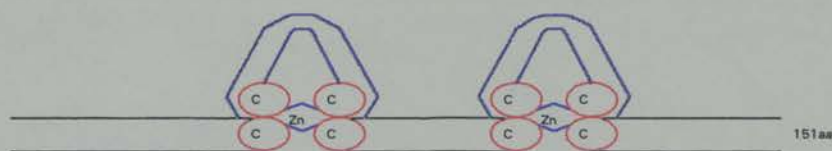


### 2.5.1 Transformation By E6 And E7 From Genital Tract Associated HPV Types

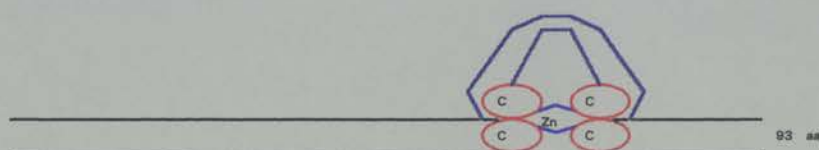
E6 and E7 constitute the major transforming proteins of HPV. The E6 and E7 ORFs encode small nuclear proteins that bind zinc through conserved cysteine motifs at their COOH-terminal end (Baker, 1987; Vousden, 1993). E6 contains two zinc binding motifs whereas E7 contains only one (Figure 1.4). Neither protein has structural similarities to any cellular genes although structural and functional similarities exist between E6 and E7 and oncoproteins encoded by other DNA viruses such as adenovirus and SV40 (Phelps et al, 1988). Although both proteins are poorly conserved in genital tract associated HPV types, E6 from high risk, EV associated HPV types show over 70% homology (Kiyono et al, 1992).

**FIGURE 1.4:- HPV E6 AND E7 PROTEINS**

#### **HPV 16 E6**



#### **HPV 16 E7**



Schematic representation of HPV 16 E6 and E7 proteins showing the cys x x cys zinc binding motifs (Vousden, 1993). The E6 protein is comprised of 151 amino acids (aa) whereas E7 contains 93aa.

DNA isolated from cervical carcinomas has been shown to transform the immortalised rodent fibroblast cell line NIH 3T3, as has DNA isolated from the high risk genital HPV types (Zur Hausen, 1989; Matlashewski, 1989; Linder & Marshall, 1990;

Munger & Phelps, 1993). Early transfection experiments demonstrated that the E6 and E7 ORFs are responsible for this *in vitro* transforming activity (Matlashewski et al, 1987; Yutsudo et al, 1988). More recent investigations into the transforming properties of single HPV genes have shown that E7 from HPV 16 is sufficient to transform NIH 3T3 cells, can immortalise primary baby rat kidney (BRK) cells and can cooperate with activated c-Ha-*ras* to fully transform these cells (Phelps et al, 1988; Munger & Phelps, 1993). The E6 proteins from HPV 16 show similar but weaker transforming activities while E6 and E7 from low risk HPV types have no, or very weak transforming potential in the same transformation assays (Storey et al, 1988; Sedman et al, 1991).

Although transfection experiments using rodent cell systems have been useful in establishing the oncogenicity of HPV, they may not be a good model for the biological events occurring in human cells. Further experiments using primary human keratinocytes have demonstrated that the introduction of HPV 16 or 18 DNA into primary human genital epithelial cells results in immortalisation, aneuploidy and reduced growth factor requirement (Hashida & Yasumoto, 1991). Expression of both E6 and E7 were required for this immortalisation, although the individual oncoproteins demonstrated reduced immortalising activity when expressed from a strong heterologous promoter (Munger et al, 1989; Hawley-Nelson et al, 1989; Sedman et al, 1991). These experiments were the first clear demonstration that E6 from high risk genital HPV types can function as an oncoprotein. HPV immortalised keratinocytes may be fully transformed by the introduction of an activated *ras* oncogene, herpes simplex virus-2 sequences or continuous passage over time (Matlashewski et al, 1987 and 1989; Munger & Phelps, 1993). Furthermore, when grown in organotypic raft culture, HPV 16 and 18 immortalised keratinocytes show defects in differentiation and morphologically resemble high grade, pre-cancerous, intraepithelial lesions (Dipaolo et al, 1989; Hurlin et al, 1991). E6 and E7 from low risk HPV types show no



transforming activities in any of these assays (Barbosa et al, 1991). These studies support the epidemiological evidence that infection with high risk HPV types gives rise to CIN and suggest that the cellular changes are a result of the activities of the E6 and E7 genes.

Research is now focused on the mechanisms by which E6 and E7 from high risk genital HPV types function. Recent studies have demonstrated that at least some of their transforming activity is related to their ability to complex with cellular proteins, in particular, the products of tumour suppressor genes.

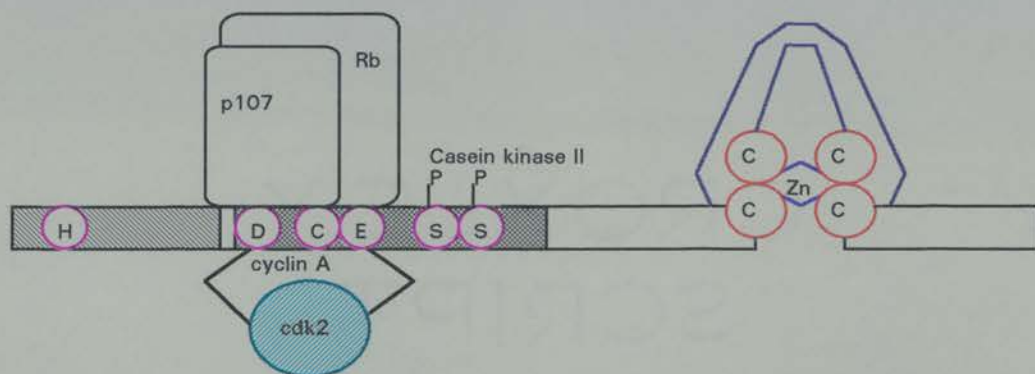
#### **2.5.2 Interaction Of E6 and E7 from Genital Tract Associated HPV Types With Cellular Proteins**

##### **E7**

The retinoblastoma (RB) gene is deleted or mutated in many human tumours or tumour cell lines and loss of this gene product (pRB) correlates with increased cell proliferation and oncogenesis (Levine & Momand, 1990). E7 from high risk genital HPV types has been shown to complex with pRB and the related protein p105 (Figure 1.5) which is thought to contribute to the development of HPV associated cancers (Munger & Phelps, 1993; Dyson et al, 1989 and 1992; Vousden, 1993). This hypothesis is supported by a small study which demonstrated an inverse relationship between the presence of RB mutations and HPV DNA in anogenital cancer cell lines, suggesting that E7 expression results in loss of RB function in HPV positive cancers (Scheffner et al, 1991). More recent studies have provided a mechanism by which E7/pRB complexes may stimulate cell proliferation and contribute to anogenital carcinogenesis. E7 has been shown to displace host cellular proteins that are usually bound to pRB such as *c-myc* and the transcription factor E2F (Rustigi, 1991;

Chellappan et al, 1992). *In vitro* expression of E7 results in an increase in free, transcriptionally active E2F which may ultimately result in increased activity of E2F promoters and unscheduled progression through the cell cycle.

**FIGURE 1.5:- ASSOCIATION OF HPV E7 PROTEIN WITH CELLULAR PROTEINS**



HPV 16 E7 and known associated cellular proteins (not to scale) (Vousden, 1993). These associations probably occur at different stages of the cell cycle. Some of the residues of E7 that have been shown to be important for transforming activity are illustrated, including the two phosphorylated serines (S) and three amino acids in the Rb binding region (D, C and E). The aspartic acid (D) in the Rb binding domain is only found in the high risk HPV E7s and substitution of this amino acid into the corresponding position in HPV 6 E7 enhances both its RB binding and transforming ability. Substitution of the second amino acid (H) at the NH<sub>2</sub> terminal of E7 destroys the transforming ability of the protein without affecting Rb binding.

Additional support for the importance of the E7/pRB interaction in oncogenesis has come from studies using directional mutagenesis. The presence of the high affinity HPV 16 E7 pRB binding site has been found to be the major determinant for the oncogenic potential of the E7 protein in transformation assays (Figure 1.5) (Heck et al, 1992). E7 mutants that do not bind pRB cannot disrupt the E2F complexes or cooperate with insulin to induce DNA synthesis (Pater et al, 1992; Takami et al, 1992). Furthermore, they have a very weak transforming activity measured by *ras* co-operation in BRK cells. The E7 proteins from the high risk genital HPV types have

been shown to bind pRB with a 10-fold higher affinity than the E7 proteins from the low risk HPV types (Munger et al, 1989; Barbosa et al, 1990; Heck et al, 1992; Munger & Phelps, 1993). This difference is due to a single amino acid sequence in the pRB binding site (Figure 1.5). A single point mutation substituting the amino acid sequence from low risk HPV E7 to an amino acid sequence common to high risk HPV E7 is able to increase the binding affinity of HPV 6 E7 to pRB and also increase its transforming activity in rodent cell assays (Heck et al, 1992; Sang & Barbosa, 1992).

As well as initiating cell proliferation by interfering with pRB-associated block on the cell cycle, E7 has also been shown to function later in the cell cycle, allowing the cell to proceed through G2 into mitosis and cell division. Although the activity of E7 necessary for this function has not yet been identified, the E7 protein has been shown to associate with a cyclin dependent kinase 2 (cdk2) specifically during this stage of the cell cycle (Figure 1.5) (Davies et al, 1993; Tommasino et al, 1993). The cyclin dependent kinases are important regulators of cell cycle progression, therefore perturbation of cyclin A and cdk2 may interrupt the normal regulation of entry into mitosis.

## E6

*In vitro* studies have demonstrated that E6 binding results in the rapid proteolytic degradation of the p53 oncoprotein through a ubiquitin dependent pathway (Werness et al, 1990; Scheffner et al, 1990 and 1992). As loss or mutation of wild type p53 contributes to the development of many human tumours, the interaction of E6 and p53 is probably an important step in the progression of most cervical malignancies. The protein interaction between E6 and p53 will be discussed further in Section 3.4 of the Introduction.

### **2.5.3 Cellular Transformation By E6 and E7 Of EV-Associated HPV Types**

In contrast to the high risk genital HPV types, a number of studies have demonstrated that E6 is the major transforming gene of EV associated HPV types and that E7 from these HPV types has little or no transforming activity.

HPV types 5 and 8 and less frequently HPV types 14, 17, 20 and 47 have been detected in SCCs from EV patients (Fuchs & Pfister, 1990). The E6 genes from HPV types 5, 8 and 47 have been shown to induce anchorage independence and reduced serum requirements in established rodent cells such as C127, 3Y1 and Rat-1 (Watts et al, 1984; Ifner et al, 1988; Deau et al, 1991; Kiyono et al, 1992). However, the resultant cells are not tumourigenic in nude mice. E6 genes from HPV types 14, 20, 21 and 25 have also been shown to partially transform the immortalised 3Y1 cell line although none were able to induce foci formation in soft agar or produce tumours in nude mice (Kiyono et al, 1992). These data suggest that E6 genes from EV associated HPV types are more weakly oncogenic than those from high risk, genital tract associated HPV types. In keeping with this hypothesis, it has been demonstrated that E6 from HPV 8 does not complex with p53 *in vitro* (Steger & Pfister, 1992). The above HPV types can be divided into two clusters depending on their malignant potential *in vivo*, their transforming activity *in vitro* and the amino acid sequence of their E6 protein (Kiyono et al, 1992). HPV types 5, 8 and 47 are in the first cluster and are classified as highly oncogenic on the basis that they are frequently found in skin cancers from EV patients (Orth, 1986). By contrast, HPV types 14, 20, 21 and 25 have infrequently (HPV 14 and 20) or never (HPV 21 and 25) been found in skin cancers and are classified as poorly oncogenic (Fuchs & Pfister, 1990). It has been demonstrated that the HPV types in the second cluster have weaker transforming activity *in vitro* and exhibit differences in the amino acid sequence of their E6 protein, largely in the second zinc finger motif (Figure 1.4). These data imply that the primary



structure of the second zinc finger of E6 may influence the transforming activity of EV-associated HPV types.

Initial investigations found that the E7 gene of HPV 8 does not transform established rodent cells *in vitro* and furthermore, does not complex with pRB (Iftner et al, 1988 and 1990). However, a more recent study demonstrated that E7 from HPV types 5 and 8 can cooperate with activated *ras* to transform primary BRK cells and complex with pRB *in vitro* (Yamashita et al, 1993). HPV 8 E7 operates at a lower efficiency than HPV 5 E7 in these transformation assays and binds pRB with a lower affinity. Because the amino acid sequence of E7 from the two HPV types is very similar, the reasons for this difference remain unclear. The discrepancy between this and other studies may be due to the level of E7 expression in the transfection assays. Further studies are therefore required to clarify the importance of the E7 gene in transformation by EV-associated HPV types.

#### **2.5.4 Genomic Integration Of HPV DNA**

The integration of HPV DNA into the cellular genome is a common event in malignant progression of genital tract lesions (Howley, 1991; Chang, 1990; Fukushima et al, 1990; Cullen et al, 1991; Lazo et al, 1992). Viral DNA is usually integrated in high grade dysplasias and carcinomas but is present in episomal form in benign and low grade pre-malignant lesions (Fukushima et al, 1990; Cullen et al, 1991). Although no specific integration sites have yet been shown in the host cell DNA, the integration site in the viral DNA appears to be highly specific, usually occurring in the E1/E2 ORFs (Lazo et al, 1992). This leads to the disruption of the E2 ORF repressor functions which may allow over-expression of the E6 and E7 oncoproteins, thus promoting the development of cervical neoplasia (Krajinovic et al, 1993). Chromosome 8q24 is a documented integration site and contains at least 4 loci implicated in tumourigenesis

including *c-myc* (Lazo et al, 1992). Disruption of cellular oncogenes provides another mechanism by which integration may contribute to cervical carcinogenesis.

Contrasting with HPV associated genital cancers, integration of viral sequences has only been reported in one EV skin cancer (Yabe et al, 1989). This was an HPV 5 infected skin cancer metastasis in which the major viral DNA species was a 40% subgenomic fragment. This fragment contained the URR and the E6 and E7 ORFs, implying that these regions are important in HPV associated skin carcinogenesis. The presence of episomal DNA with deletions has been more frequently reported in EV cancers, usually disrupting the late region (Yabe et al, 1989; McDeau et al, 1991). These modifications may result in the deregulation of viral gene expression, playing a similar role to integration in the progression of EV tumours.

#### **2.5.5 Additional Events Required For Malignant Progression**

Several lines of evidence suggest that additional events are required for the development of HPV-associated anogenital and skin cancers. First, there is a long latency period between infection and the development of malignancy (Howley, 1991). Second, a co-carcinogen is easily identified in many PV related cancers such as UV radiation in EV skin cancers and bracken fern in bovine alimentary tract carcinomas (Fuchs & Pfister, 1990; Pennie & Campo, 1992; Schneider, 1993). Finally, HPV types 16 and 18 require activated *ras* or other events for complete cellular transformation *in vitro* (Phelps et al, 1988; Munger & Phelps, 1993). Further investigations into the genetic events associated with these additional factors may provide insight into how they cooperate with HPV to bring about carcinogenesis.



### **3: THE P53 TUMOUR SUPPRESSOR GENE**

#### **3.1 P53 AND CARCINOGENESIS**

##### **3.1.1 p53 Mutations And 17p Deletions In Sporadic Human Tumours**

p53 gene mutations are the most commonly observed genetic lesion in human cancers, occurring in over 50% of colon (Nigro et al, 1989; Baker et al, 1989 and 1990; Cunningham et al, 1992), lung (Nigro et al, 1989; Iggo et al, 1990; Henzel et al, 1992), breast (Nigro et al, 1989; Varley et al, 1991), bladder (Sidransky et al, 1991), hepatocellular (Brassac et al, 1991) and stomach (Tamura et al, 1991) carcinomas, which account for most neoplasms observed in humans. Deletions of the short arm of chromosome 17 (17p), harbouring the p53 locus, are also a frequent event in human tumours and have been demonstrated in over 60% of colon, breast, lung, ovarian, cervical, adrenocortical, bone and bladder cancers (Mackay et al, 1988; Delattre et al, 1989; Weston et al, 1989; Baker et al, 1990; Tsai et al, 1990; Carder et al, 1991; Eccles et al, 1992; Busby-Earle et al, 1993). In 75-80% of cases, 17p deletions and p53 point mutations occur simultaneously, resulting in inactivation of both alleles of the p53 gene (Nigro et al, 1989; Baker et al, 1990; Levine et al, 1991; Stratton et al, 1992). Allelic losses, accompanied by inactivation of the remaining allele through localised point mutation, usually indicates the presence of a tumour suppressor gene (Knudson, 1971). Therefore, these data indicate that p53 acts as a tumour suppressor gene and that inactivation of this gene is a critical event in human carcinogenesis.

##### **3.1.2 Germline p53 Mutations**

Three systems in which there are germline abnormalities of the p53 gene, support the hypothesis that mutations in this gene are a causal event in carcinogenesis. Li-Fraumeni syndrome is a rare, familial predisposition to cancer that is transmitted in an autosomal dominant manner (Li & Fraumeni, 1969). The syndrome is characterised clinically by sarcomas in children with female relatives who have a high incidence of breast cancer.

Leukaemia, brain, lung and adrenal cortical tumours constitute less common features of the syndrome. Li-Fraumeni patients carry one wild type and one mutant p53 in their genome which does not appear to interfere with the developmental processes or normal cellular functions (Malkin et al, 1990; Strivastava et al, 1990; Borresen et al, 1992). In cancers, the mutant allele is retained and expressed, but the wild type allele is most commonly lost via deletion or, in a minority of cases, gene conversion. Thus, it has been proposed that the germline mutations in p53 predispose Li-Fraumeni patients to cancer since somatic mutation/deletion need only occur in one allele for a cell to escape growth controls imposed by the tumour suppressor gene. The distribution of mutations throughout the p53 gene in Li-Fraumeni patients is similar to that seen in sporadic tumours, although, there is an increase in mutations in conserved region IV of the gene (see Section 3.2 of the Introduction) (Soussi et al, 1990; Borresen et al, 1992).

Transgenic mice and p53 gene knockout mice have been used as model systems to investigate the role of p53 in developmental processes and in carcinogenesis (Donehower et al, 1992; Harvey et al, 1993; Purdie et al, 1994).. These studies demonstrate that overexpression of the p53 val<sup>135</sup> mutation in mice does not disrupt the development of the animal but leads to (in 20% of cases) an excess of lung adenocarcinomas, osteosarcomas and lymphomas, which is similar to the spectrum of cancers seen in Li-Fraumeni patients (Laviguer et al, 1989). There is a long latent period prior to cancer development in these transgenic mice, suggesting that overexpression of mutant p53 alone is not sufficient to induce malignancies. Complete knockout of the p53 gene has been achieved in mouse lines by gene targeting techniques (Donehower et al, 1992; Harvey et al, 1993; Purdie et al, 1994). p53 null mice also develop normally, indicating that the p53 gene is dispensable for mouse development, however, the animals are susceptible to spontaneous tumours with nearly 75% of homozygous mice developing multiple cancers by 6 months of age

(Donehower et al, 1993). p53 knockout mice appear to be particularly susceptible to the development of T cell lymphomas, but a wide variety of cancers have been demonstrated. Heterozygous mice, with one wild type p53 allele, also develop spontaneous tumours but with a delayed onset compared to homozygotes and osteosarcomas and soft tissue sarcomas predominate in these mice, reminiscent of Li-Fraumeni families (Harvey et al, 1993). Interestingly, a number of homozygous mice die of unresolved infections, suggesting that they may have a defect in their immune systems (Purdie et al, 1994).

### **3.1.3 Accumulation And Mutation Of p53 In Cutaneous Carcinogenesis**

p53 accumulation has previously been demonstrated in both pre-malignant and malignant epidermal lesions from ICPs. However, the prevalence has been somewhat inconsistent, ranging from 0-55% of solar keratoses, 17-80% of Bowens disease and 15-56% of cutaneous SCCs (Gusterson et al, 1991; McGregor et al, 1992; Ro et al, 1993; Helander et al, 1993; Nagano et al, 1993). More direct methods have also been used to investigate the prevalence of mutated p53 in pre-malignant and malignant cutaneous lesions from ICPs but once again, the detection level varies between studies: Brash et al (1991) detected p53 gene mutations in 14/24(58%) cutaneous SCCs whereas Pierceall et al (1991a) and Moles et al (1993) detected mutated p53 in 2/10 (20%) and 2/13(15%) cutaneous SCCs respectively. p53 gene mutations have also been demonstrated in 48% of 20 Bowen's disease (Campbell et al, 1993a). The distribution of p53 mutations observed in these investigations was similar to the mutational spectra observed for other cancer types (see Section 3.5.1 of the Introduction).

### **3.2 THE GENE FOR P53 AND ITS TRANSCRIPT**

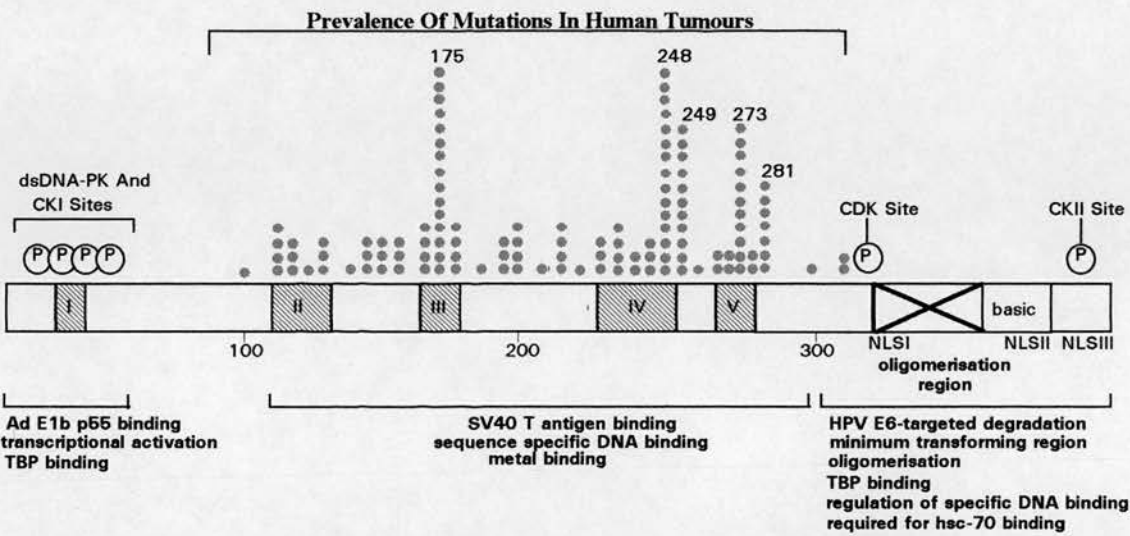
The p53 gene encompasses 16-20 KB of DNA on the short arm of chromosome 17 at 17p13.1 (Baker et al, 1989). Mouse, human and *Xenopus laevis* p53 genes have all

been fully sequenced and comprise eleven exons interrupted by 10 introns (Soussi et al, 1987 and 1990). p53 cDNAs have also been cloned from rat, chicken, monkey, hamster and trout genes, although no p53 related sequences have yet been identified in invertebrate species. Northern blot analysis reveals that the mRNA for human p53 is 2-3KB long and is most abundant in undifferentiated stem cells, spleen cells, cells undergoing rapid embryonic development and other rapidly proliferating cell types (Bendori et al, 1987; Khochbin & Lawrence, 1989; Schmid et al, 1991). The levels of p53 mRNA have been shown to be elevated in some tumours but are down-regulated in tumour cells induced to differentiate (Bendori et al, 1987). These data suggest that the proliferative state of the cell correlates with the levels of p53 mRNA.


The human p53 protein comprises 393 amino acids (approximately 53 KDa) and structurally resembles a transcription factor (Figure 1.6) (Donehower & Bradley, 1993). The protein can be divided into three domains based upon analysis of the primary amino acid sequence (Soussi et al, 1990). The first 75-80 amino acids comprise the acidic NH<sub>2</sub> terminus which is highly charged and contains the transcription activation sequence (Levine & Momand, 1990). The central portion of the molecule is a proline rich domain which contains 5 highly conserved regions (domains I-V in Figure 1.6) that share over 90% homology between species (Soussi et al, 1987). The majority of missense p53 mutations are in these conserved regions, implying that they may be of particular functional importance (Soussi et al, 1990; Bennet et al, 1992). This hypothesis is supported by the recent discovery that the central conserved core of p53 represents the DNA binding domain of the protein (Cho et al, 1994; Vogelstein & Kinzler, 1994). The COOH terminus is rich in basic amino acids and contains oligomerisation signals which have been shown to be important in the growth suppressor activities of p53 (Prives & Manfredi, 1993). Three nuclear localisation signals (NLS) are also present in the COOH-terminus of p53 (Rotter et al, 1993).



**FIGURE 1.6: STRUCTURAL DOMAINS ON THE HUMAN P53 PROTEIN**



Structural domains on human p53 (Prives & Manfredi, 1993). The shaded boxes containing Roman numerals represent the 5 conserved domains of p53. The three nuclear localisation signals (NLS) are also shown. Indicated above are the known phosphorylation sites on p53 (P): the two carboxy-terminal sites have been shown to be phosphorylated by cdc2 and cdk2 kinases (CDK site) or casein kinase II (CKII site), putative kinases for the amino four sites include double-stranded DNA protein kinase (dsDNA-PK) and casein kinase I (CKI). The red dots in the centre show sites that are commonly mutated in human tumours, the hot spots are identified by amino acid number. TBP is the TATA box binding protein; hsc 70 is heat shock protein 70.

 indicates oligomerisation sequence

**3.3 BIOLOGICAL FUNCTIONS OF WILD TYPE P53**

**3.3.1 p53 Acts As Tumour Suppressor**

The expression of wild type p53 in rat embryo fibroblasts transformed by mutant p53 (or other oncogenes such as c-myc, E1A and E7) plus activated *ras* actively suppresses transformation, as measured by numbers of transformed foci (Eliyahu et al, 1989; Finlay et al, 1989; Chen et al, 1990; Yin et al, 1992; Takahashi et al, 1992). The rare foci that form under these conditions do not express the exogenous wild type p53 gene. In addition to suppressing transformation of rodent cells, the wild type p53 gene



is capable of reverting the transformed phenotype of human colon (Baker et al, 1990), bladder (Chen et al, 1990), brain (Mercer et al, 1990) and bone (Diller et al, 1990) cancer cell lines. These *in vitro* studies provide strong evidence that p53 functions as a tumour suppressor gene and suggest that this gene is selected against during transformation. The finding that both p53 alleles are inactive in the majority of human tumours (see Section 3.1.1 of the Introduction) and in mouse erythroleukaemias induced by Freund virus, supports the hypothesis that p53 acts as a tumour suppressor gene (Mowat et al, 1985; Nigro et al, 1989; Baker et al, 1990; Levine et al, 1991 and 1993; Stratton, 1992) .

### **3.3.2 p53, DNA Damage, Apoptosis And The Cell Cycle**

Exposure to DNA damaging agents contributes to the development of many human cancers, especially those of organs frequently exposed to carcinogens such as the skin. An important cellular response to DNA damage is inhibition of replicative DNA synthesis and transient arrest in G1-S phase of the cell cycle, presumably to allow time for effective DNA repair (Hartwell, 1992). A number of human tumour cell lines have been shown to arrest in the G1 phase of the cell cycle following overexpression of p53 (Baker et al, 1990; Chen et al, 1990; Diller et al, 1990). It has also been demonstrated, both *in vitro* and *in vivo*, that cellular levels of p53 are elevated following exposure to DNA damaging agents (such as ionising radiation, mitomycin C, etoposide and DNA restriction enzymes) and that these elevated levels of p53 temporarily correlate with a transient G1 arrest (Kastan et al, 1991; Keurbitz et al, 1992; Hall et al, 1993). Cells that are devoid of p53 or express mutant p53 fail to block growth in the G1 phase of the cell cycle following gamma irradiation, however, the restoration of a wild type p53 allele to these cells restores the G1 arrest response (Yin et al, 1992).

Another important cellular response sometimes induced by DNA damaging agents is cell death by apoptosis. Apoptosis is a distinct, genetically programmed pathway of

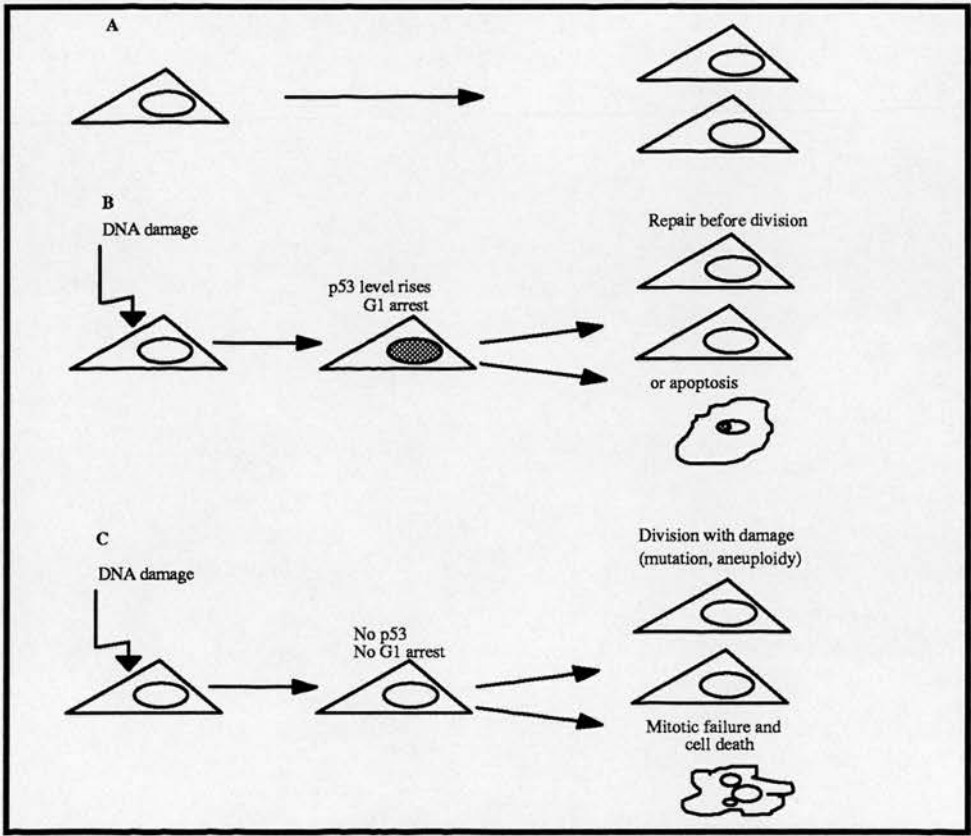


cell death that plays an important role in normal development and underlies processes such as organogenesis, tissue homeostasis and the deletion of autoreactive clones from the immune system (Williams & Smith, 1993). Apoptosis may also play a role in the kinetics of tumour growth (Arends et al, 1994; Arends & Harrison, 1994). Overexpression of wild type p53 in a murine myeloid cell line results in increased apoptosis as indicated by characteristic cell morphological changes and DNA fragmentation (Yonish-Rouach et al, 1991). Furthermore, a colon carcinoma cell line expressing wild type p53 under an inducible promoter forms tumours in the absence of the inducer but when the inducer is present, the tumours regress until they are totally eliminated (Shaw et al, 1992). The cells induced to die have characteristics of apoptotic cells. These data indicate that wild type p53 induces apoptosis in some circumstances (Lane, 1993). Studies using p53 gene knockout mice have recently demonstrated that thymocytes with no endogenous p53 are highly resistant to gamma irradiation-induced apoptosis, both *in vivo* and *in vitro* (Clarke et al, 1993; Lowe et al, 1993a). However, these cells undergo a normal apoptotic response to treatment with glucocorticoids, indicating that there are p53 dependent and independent pathways for apoptosis (Clarke et al, 1993). Anticancer agents are thought to act by inducing DNA damage and apoptosis in actively proliferating cells. The data acquired from the p53 null mice imply that tumour cells with only one or no copies of the wild type p53 gene may be more resistant to the apoptotic response induced by these agents (Clarke et al, 1993; Lowe et al, 1993b). This suggests a mechanism whereby tumour cells can acquire cross resistance to anticancer agents which has important implications for cancer therapy.

On the basis of these data and the finding that p53 is not required for normal cell cycle control, a model has been put forward for the function of wild type p53 (Figure 1.7) (Lane, 1992 and 1993). It has been proposed that wild type p53 acts as a "molecular policeman" monitoring the integrity of the genome in periods of cell stress. DNA

damage results in the accumulation of p53 and the inhibition of DNA synthesis to allow time for its repair. In circumstances of extensive, irreparable DNA damage, p53 induces cell death by apoptosis. Inactivation of wild type p53 leads to the loss of control of this cell cycle checkpoint and permits the development of genomic instability which is a characteristic of transformed cells.

**FIGURE 1.7:- A MODEL FOR THE FUNCTION OF p53**



A model for the function of p53 (Lane, 1992 and 1993). **A:** normal cell division for which p53 is not required. **B:** the genome guarding function of p53 in a normal cell is induced in response to DNA damage. **C:** cells in which the p53 pathway is inactivated by mutation or cellular or viral proteins replicate damaged DNA, resulting in mutation, aneuploidy and cell death. Malignant clones may arise from cells that survive with damaged DNA.

Support for this hypothesis has come from the finding that primary fibroblasts from Li-Fraumeni patients, passaged until they are homozygous for mutated p53, have increased genetic instability (measured as a function of gene amplification) compared to fibroblasts that contain one or two copies of wild type p53 (Bischoff et al, 1990a). Similarly, fibroblasts from mice with no functional p53 amplify genes at a high frequency whereas heterozygous and wild type cells do not (Tsukada et al, 1993). These data are a direct indication that lack of p53 results in genetic instability. Patients with the rare disorder ataxia-telangiectasia are hypersensitive to ionising radiation, have a pre-disposition to cancer development and fail to block growth in the G1 phase of the cell cycle following DNA damage (Khanna & Lavin, 1993). Kastan and colleagues (1992) demonstrated that cells from these patients lack the ionising radiation-induced increase in p53 that is commonly observed in normal cells, thus providing further evidence for a link between DNA damage, p53, genetic instability and cancer.

### **3.3.3 p53 And Differentiation**

A number of studies have suggested that p53 may be involved in differentiation in some cell types (Rotter et al, 1993). When wild type p53 is expressed in a B cell line that has no endogenous p53, the cells proceed to a more advanced stage of B cell differentiation, as indicated by increased expression of cytoplasmic IgM as well as other B cell differentiation markers (Shaulsky et al, 1991a and b). When transplanted into mice, B cells expressing wild type p53 give rise to tumours at a lower frequency and of a smaller size compared to those arising from the parental cells with no endogenous p53. Furthermore, tumours expressing wild type p53 show terminal cell differentiation and express high levels of heavy and light immunoglobulins, whereas tumours arising from p53 non-producers are highly proliferative and are more rapidly lethal to the host (Shaulsky et al, 1991b). Transgenic mice bearing a p53 responsive promotor adjacent to a chloramphenicol acetyltransferase (CAT) reporter have

elevated CAT expression in the testes, implicating a role for p53 in sperm cell differentiation and maturation (Rotter et al, 1993). Transfection of wild type p53 into human osteosarcoma Soas-2 cells, followed by injection of these cells into nude mice, results in metastases consisting almost entirely of differentiated bone tissue (Prives & Manfredi, 1993). The demonstration of an upregulation of p53 mRNA in myoblasts induced to undergo terminal differentiation suggests that p53 may also be involved in muscle cell differentiation (O'Halevy, 1993). Finally, it has been observed that p53 mRNA is detected in mid-gestation embryos, particularly in regions of accelerated cell differentiation activity, and that *Xenopus laevis* oocytes and early embryos, contain substantial quantities of p53 protein and RNA (Rogel et al, 1985; Prives & Manfredi, 1993). These experiments suggest that p53 may be involved in differentiation and developmental processes. However, B cell differentiation and spermatogenesis both involve DNA rearrangements followed by DNA repair. Therefore, p53 may have a role in these differentiation processes through the induction of G1 arrest to allow time for this repair. The notion that p53 is essential for cell differentiation and development *in vivo* is also challenged by the existence of the p53 null mouse (Donehower et al, 1992; Purdie et al, 1994).

### **3.4 REGULATORY PATHWAYS OF WILD TYPE P53**

#### **3.4.1 p53 Is A Transcriptional Transactivator And Repressor**

Recent studies have demonstrated that p53 is a sequence specific DNA binding protein with transcriptional activator and repressor properties (Kern et al, 1991; Funk et al, 1992; Farmer et al, 1992; Unger et al, 1992; El-Diery et al, 1992; Tarunina et al, 1993; Chumakov et al, 1993). The first demonstration of mammalian gene regulation by p53 was the muscle specific creatine phosphokinase (mck) gene (Weintraub et al, 1991). p53 has subsequently been shown to bind to and transactivate a number of other mammalian genes including the murine double minute-2 (mdm-2) gene (see Section 3.4.2 of the Introduction) (Liu et al, 1993; Shilo et al, 1993; Juven et al, 1993). Wild



type p53 can also negatively regulate a wide variety of viral and cellular promoters such as those from c-fos, beta actin, c-jun, IL-6, heat shock protein (hsp) 70, Rb, multidrug resistance gene, Rous sarcoma virus, HIV, herpes simplex virus-1, SV40 and cytomegalovirus (Donehower & Bradley, 1993). p53 appears to repress transcription by an indirect mechanism, possibly through the inactivation of general transcription factors (see Section 3.4.2 of the Introduction). It has recently been demonstrated that p53 can transactivate its own promoter and sequences similar to the p53 consensus binding site appear to be critical for this autoregulation (Deffie et al, 1993).

It is likely that all the observed biological functions of p53 are mediated by the regulation of genes at the transcriptional level. Support for this hypothesis comes from the finding that viral oncoproteins that bind p53 and induce cellular transformation, also inhibit p53 mediated transcriptional transactivation (see Section 3.5.3 of the Introduction) (Yew & Berk, 1992; Lechner et al, 1992; Moran, 1993; Kesis et al, 1993). Mutant viral proteins that are defective in transformation are also defective in blocking p53 induced transcription. These data suggest that transactivation is necessary for p53 to function as a tumour suppressor. Increased expression of the GADD45 damage response gene usually occurs following exposure to DNA damaging agents (Kaufman & Kaufman, 1993). Therefore, the demonstration that p53 binds strongly to a conserved element within the GADD45 gene and upregulates expression of this gene provides further evidence that p53 may induce G1 arrest following DNA damage through transactivation of other genes (Kastan et al, 1992; Donehower & Bradley, 1993). p53 is also able to directly induce expression of WAF1, which is a potent inhibitor of cyclin dependent kinases and inhibits growth of human tumour cell lines and normal diploid fibroblasts, further supporting this hypothesis and suggesting that p53 functions as part of a complex pathway of cell cycle control (El-Deiry et al, 1993 and 1994; Harper et al, 1993). However, the suggestion that p53 functions only

through activation of cell cycle control genes is challenged by the finding that wild type p53 can induce apoptosis in immortalised pituitary cells in the presence of large doses of transcription and translation inhibitors, implying that p53 has other functional properties (Caelles et al, 1994).

#### **3.4.2 p53 Protein Interacts With Cellular Proteins**

The mdm-2 oncogene product was the first cellular protein found to complex with p53 *in vitro* (Momand et al, 1992; Oliner et al, 1992). This protein binds to the p53 protein adjacent to its transactivation domain and inhibits the ability of p53 to activate transcription, perhaps by concealing its transactivation domain from the cell's transcriptional machinery (Momand et al, 1992, Oliner et al, 1993). The level of mdm-2 protein/p53 protein complex increases in the G1 phase of the cell cycle, implying that the product of the mdm-2 gene may regulate p53 at the protein level, which in turn regulates mdm-2 gene expression (see above) (Olson et al, 1993). On the basis of this data, an autoregulatory model involving the mdm-2 gene has been proposed for p53.

CAAT binding protein (CBP), E6-Ap, replication protein A (RPA), Sp1, TATA binding protein (TBP), TFIID and the Wilms tumour gene product have more recently been shown to form protein complexes with p53 (Pietenpol & Vogelstein, 1993). The interactions between p53 and the general transcription factors CBP, TBP and TFIID are of particular interest because these may provide an indirect mechanism for p53 mediated regulation of transcription (Liu et al, 1993). Further experiments are required to determine the biological relevance of interactions between p53 and cellular proteins.

#### **3.5 MODIFICATION OF WILD TYPE P53**

Modification of wild type p53 commonly occurs through 17p deletions and/or p53 point mutations in human tumours (see Section 3.1.1). However, it is now known that



interactions with cellular or viral proteins may also result in inhibition of the normal functions of wild type p53.

### **3.5.1 Distribution And Nature Of p53 Gene Mutations In Human Tumours**

The majority of p53 mutations are missense single base substitutions resulting in replacement of one amino acid by another in the p53 protein (Prives & Manfredi, 1993). These mutations are not distributed randomly across the p53 gene but are found clustered between codons 120-290 out of 393 amino acid residues (Figure 1.6). Mutations are particularly prevalent in four regions of the protein product (residues 117-142, 171-181, 234-258 and 270-286) which correspond with four of the most highly conserved regions of the gene amongst several different species (see Section 3.2 of the introduction) (Soussi et al, 1990). In fact, about 40% of the missense mutations in carcinomas occur in one of five "hotspot" codons (residues 175, 249, 273, 281 or 284) which appear to be specific for different cancer types. It has recently been demonstrated that these frequently mutated residues are all at or near the p53 protein-DNA interface suggesting that they function through altering the DNA binding properties of wild type p53 (Cho et al, 1994). The nature of p53 mutations also appears to be tumour specific and related to the mutagen involved. For example, G to T transversion mutations, which are known to be induced by benzopyrene in cigarette smoke, are present in lung cancers but are never observed in colorectal cancers where different mutagens act upon the DNA (Chiba et al, 1990; Harris, 1991). Similarly, CC-TT double base mutations, which are almost unique to UV induced DNA damage, have only been demonstrated in skin cancers (Brash et al, 1991). However, if the narrow distribution and nature of the p53 mutations were simply a function of the type of mutagen involved, a broader spectrum of mutations would be expected in human cancers. Alternatively, mutations in these "hotspot" regions may be selected for in human cancers, suggesting that such cancers do not just result from loss of function mutations, but that altered p53 proteins contribute some functional advantage to the

tumour. This hypothesis has now been tested, and indeed, mutated p53 proteins can confer new phenotypic properties on cells lacking endogenous p53 (Dittmer et al, 1993).

### **3.5.2 Mutant p53 And Transformation**

Early studies demonstrated that transfection of mutant forms of p53 into rodent fibroblasts rapidly induced an immortal phenotype in the transfected cells (Hinds et al, 1989; Zambetti et al, 1992; Zambetti & Levine, 1993). Mutant p53 can also co-operate with activated *ras* to fully transform primary rat embryo fibroblasts in culture. The mechanisms underlying transformation by mutant p53 are presently unknown, however, a number of suggestions have been made. Mutant p53 proteins have an altered conformation when compared to wild type protein and mutant forms of p53 have been shown to form heterologous complexes with wild type p53, driving the wild type protein into a mutant conformation (see Section 3.5.3 of the Introduction) (Kraiss et al, 1988; Milner et al, 1991; Milner & Medcalf, 1991; Stenger et al, 1992). Therefore, one suggestion is that mutant p53 transforms cells through binding and inactivating the wild type protein. The selective pressure in human cancer for mutant p53 rather than no p53 at all suggests that mutant p53 may contribute to the gain of some growth altering function rather than inactivating wild type p53 activity. Evidence for gain of function mutations in p53 comes from the finding that mutant forms of p53 transform immortal murine fibroblasts, lacking endogenous p53, to a fully tumourigenic cell line (Dittmer et al, 1993). These cells do not express wild type p53, therefore, mutant p53 cannot be functioning in a dominant negative manner in these assays.

### **3.5.3 Conformation And Stability Of Wild Type And Mutant p53**

In non-transformed cells, wild type p53 is rapidly degraded via a ubiquitin dependent pathway. However, missense mutations of the p53 gene uniformly result in stabilisation

of the p53 protein which increases in its half life from 5-40min (wild type) to several hours (mutant forms) (Oren et al, 1981; Milner & Cook, 1986; Finlay et al, 1988). Consequently, mutant p53 accumulates within transformed cells allowing its detection by immunocytochemical techniques (Dippold et al, 1981; Rotter et al, 1981; Benchimol et al, 1982; Jenkins et al, 1984; Bartek et al, 1991; Montenarh, 1992; Wynford-Thomas, 1992). Wild type p53 is usually present in cells at such low levels that it cannot be detected by this technique. Thus, immunocytochemical demonstration of p53 in tissues may indicate the presence of mutated p53, even if conformation-independent antibodies (those that bind to wild type and mutated p53) are used. However, non-transformed cells that have recently been exposed to DNA damaging agents, such as cutaneous keratinocytes exposed to UV radiation, also exhibit immunocytochemically detectable levels of wild type p53 (Hall et al, 1993). Immunocytochemical methods have been used extensively to investigate human malignancies for p53 gene mutations and it has been found that accumulation of p53 is a common event in a wide variety of human cancers (Cattoretti et al, 1988; Iggo et al, 1990; Bartek et al, 1990 and 1991; Rodriguez et al, 1990; Purdie et al, 1991; Scot et al, 1991; Varley et al, 1991; Bodner et al, 1992; Thomson et al, 1992). In the majority of these studies, immunoreactive p53 was confined to malignant cells which supports the hypothesis that immunocytochemically detectable p53 indicates an abnormality in the p53 pathway.

The missense mutant forms of p53 have an altered conformation when compared to the wild type protein. The monoclonal antibody PAb246 binds to wild type p53 at an epitope between amino acid residues 88 and 109 but fails to bind to mutant forms of the protein (Yewdell et al, 1986). Conversely, monoclonal antibody PAb240 binds to many diverse mutant forms of the p53 protein at an epitope between residues 206 and 211 but this antibody fails to bind to the wild type protein (Gannon et al, 1990). A number of groups have demonstrated concordance in the cells within a tumour that

stain with the mutant specific antibody PAb240 and the conformation-independent antibody PAb1801 which also supports the contention that immunocytochemically detectable p53, using a conformation-independent antibody, is indicative of an abnormality in the p53 pathway (Bartek et al, 1990; Rodriguez et al, 1990; Purdie et al, 1991; Varley et al, 1991; Vojtesek et al, 1992; Burns et al, 1993).

#### **3.5.4 Cellular Proteins Inactivate Wild Type p53**

The product of the *mdm-2* gene binds and inactivates wild type p53 (Momand et al, 1992; Oliner et al, 1992 and 1993). Amplification of this gene has been demonstrated in over one-third of human sarcomas and in the majority of these sarcomas, the p53 gene is not mutated (Oliner et al, 1992). Furthermore, transfection of *mdm-2* plus activated *ras* plus wild type p53 into primary rat embryo fibroblasts results in the formation of transformed foci expressing wild type p53 (Finlay et al, 1993). Therefore, amplification of *mdm-2* in sarcomas may overcome the growth suppressive properties of wild type p53 and thus contribute to carcinogenesis. It is likely that other cellular proteins inactivate wild type p53 during carcinogenesis, although these proteins have yet to be identified.

#### **3.5.5 Viral Proteins Inactivate Wild Type p53**

In addition to complex formation with cellular proteins and gene deletions and mutations, wild type p53 may be inactivated by complex formation with the transforming proteins of some DNA tumour viruses (Yew & Berk, 1992; Lechner et al, 1992; Moran, 1993; Kesis et al, 1993). It has been demonstrated that the SV40 large T antigen, the adenovirus E1B protein and the E6 protein from the high risk HPV types all complex with p53. However, these proteins bind to the p53 protein at different regions (Figure 1.6) and appear to inactivate p53 by different mechanisms.



### SV40 large T antigen

p53 was first discovered as a cellular protein because it formed a tight complex with the SV40 large T antigen and was therefore co-immunoprecipitated, with anti T antibodies, from extracts of SV40 transformed cells (Lane & Crawford, 1979; Linzer & Levine, 1979). It has recently been demonstrated that large T antigen has the ability to block the binding of p53 to DNA, which suggests that the oncogenic potential of T antigen is mediated by its ability to inhibit transcriptional regulation by p53. SV40 transformed cells have increased cellular levels of wild type p53, therefore, it was originally presumed that binding of T antigen to p53 stabilised the p53 protein and increased its half life in the cell (Montenarh et al, 1986; Kraiss et al, 1988). However, more recent experiments have demonstrated that the half lives of free p53 and T antigen-complexed p53 are identical in SV40 transformed cells (Ludlow, 1993). This suggests that the stabilisation of p53 may result from factors other than T antigen binding, possibly as part of a cellular mechanism whereby the cell attempts to resist viral transformation. The reduced p53 turnover may also be attributed to metabolic changes associated with viral transformation.

### Adenovirus E1B

Adenovirus E1B encodes two protein products of 55KD and 19KD which are important for adenovirus transforming activity. The 55KD protein binds the acidic amino terminus of p53, thus blocking p53 mediated transcriptional transactivation (Yew & Berk, 1992; Moran, 1993). E1B transformation defective mutants are also defective in blocking p53 induced transcription, which suggests that this function of p53 is integral to the mechanism by which the 55KD protein contributes to transformation (Yew & Berk, 1992). Stable complexes between 55KD and p53 have also been shown to inactivate p53 by sequestering the protein outside the nucleus. More recent studies suggest that one of the main functions of the E1B protein in



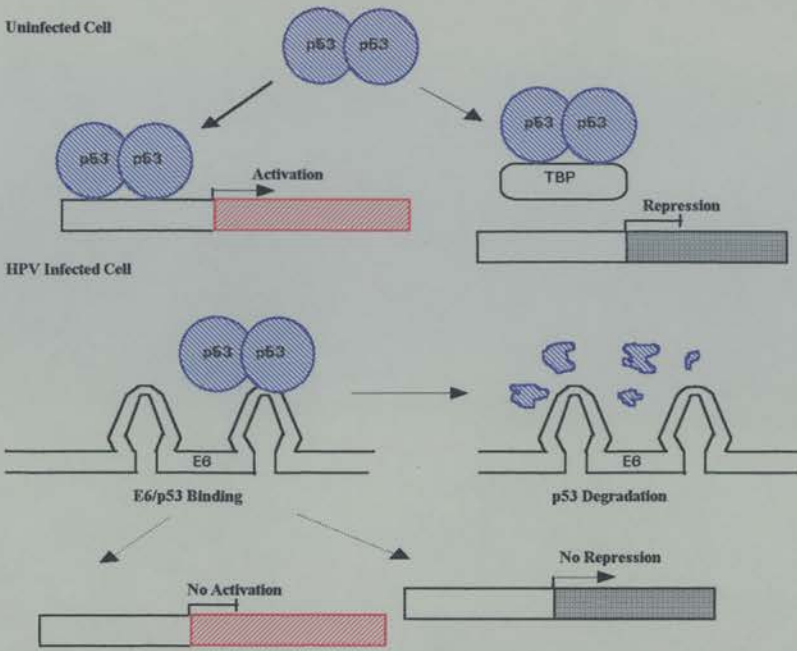
transformation is to block apoptosis that may otherwise be induced by E1A, the other major transforming protein of adenovirus (Debbas & White, 1993).

### HPV E6

In contrast to adenovirus and SV40 induced stabilisation of p53, *in vitro* studies have demonstrated that binding of HPV 16 or 18 E6 results in the rapid, proteolytic degradation of the p53 protein via a ubiquitin dependent pathway (Figure 1.8) (Werness et al, 1990; Scheffner et al, 1990 & 1992; Ciechanover et al, 1991; Vousden, 1993). *In vivo* studies have subsequently found that human epithelial cells expressing E6 also show a marked reduction in the stability of newly synthesised, endogenous p53 (Band et al, 1991). E6 degradation of p53 requires two independent domains of the E6 protein: a C-terminal region of E6 is important for p53 binding and N-terminal sequences are necessary to direct p53 degradation (Crook et al, 1991c; Lechner et al, 1992; Scheffner et al, 1992). The precise mechanism by which E6 targets p53 for degradation has not yet been established, although binding to p53 seems to be essential in this process. Proteolysis of p53 usually occurs via a ubiquitin dependent pathway, therefore, it may be that E6 binding enhances this normal pathway. Alternatively, E6 may direct degradation by a novel ubiquitin dependent pathway (Vousden, 1993). Another cell encoded protein, E6-AP, has been identified which associates with E6 and is necessary for p53 degradation to occur (Huibregtse et al, 1991). The involvement of cellular factors suggests that there may be cell-type dependent differences in the effect of E6 on p53.

Similar to large T antigen and E1B, E6 has the ability to inhibit p53 mediated transcriptional transactivation and repression (Crook et al, 1991; Lechner et al, 1992; Kessis et al, 1993). It has been demonstrated that these functions of p53 are required for p53 induced inhibition of cell proliferation. Therefore, the interaction between E6 and p53 probably represents an important step in cervical carcinogenesis. Support for

**FIGURE 1.8:- HPV E6 INACTIVATES THE NORMAL FUNCTIONS OF WILD TYPE P53**



Consequences of E6 expression on p53-mediated transcriptional control. In uninfected cells, p53 regulates gene expression through transcriptional activation and repression. In HPV infected cells, wild type p53 is inactivated through degradation, thus preventing p53 mediated transcriptional control. TBP = TATA box binding protein.

this hypothesis originally came from studies demonstrating that anogenital carcinomas, and derived cell lines, that are not infected by HPV have mutations in the p53 gene, whereas most of those cancers that are infected with HPV types 16 and 18 show wild type p53 (Scheffner et al, 1991; Crook et al, 1991a and 1992). However, subsequent studies have found that this inverse relationship is not absolute (Cooper et al, 1993; Busby-Earle et al, 1993). p53 mutations have been demonstrated in metastases from cervical cancers showing low levels of mutated p53 (Prives & Manfredi, 1993; Mitranirosenbaum & Tsvieli, 1994). This suggests that mutation of p53 contributes to the further progression of cervical cancers that initially contained wild type p53, inactivated at the protein level by E6

E6 proteins from the low risk HPV types 6 and 11 have also been shown to bind to and degrade p53 *in vivo* (Band et al, 1991). However, *in vitro* assays have produced conflicting reports with some suggesting that these E6 proteins bind to p53 but do not direct its degradation, while other reports suggest that the E6 proteins from the low risk HPV types do not bind p53 but do enhance its degradation (Werness et al, 1990; Crook et al, 1991c; Lechner et al, 1992). It seems likely that E6 proteins of both high and low risk HPV types bind and mediate degradation of p53, but with significantly different efficiencies.

### **3.6 SUMMARY**

The development of skin cancer following renal transplantation is a serious clinical problem, even in countries such as Britain where patients are exposed to only mild to moderate levels of UV radiation. The number of patients receiving such transplants is increasing and improvements in immunosuppression have produced significantly longer graft survival times. Therefore, the incidence of immunosuppression related cutaneous disease is set to rise. Thus, investigations into the factors involved in the development of skin neoplasms in RARs, and their associated genetic events, are of considerable importance.

The possible association between HPV infection and the development of skin cancer in RARs was initially suggested by observations in the rare, inherited skin disease EV. Similar to transplant patients, EV patients have a decrease in cell mediated immunity and an increase in VWs and SCCs on sun exposed body sites. Over 90% of cutaneous SCCs from EV patients are infected with HPV types 5 or 8. The clinical and histological overlap between VWs and SCCs in RARs also provides supportive evidence to suggest that HPV contribute to the carcinogenic process of RARs. However, reports on the prevalence and types of HPV DNA present in cutaneous neoplasms from RAR have been inconsistent and the role of the virus in the

carcinogenic process has yet to be fully characterised. In particular, it is not known whether HPV contribute directly to skin carcinogenesis by supplying viral oncogenes that induce neoplastic change or indirectly by promoting cell proliferation.

Although a number of putative aetiological factors have been implicated in cutaneous carcinogenesis of RARs, little is known of the associated genetic events and whether these differ in RARs and ICPs. Mutations in the p53 tumour suppressor gene are the most commonly observed genetic lesion in human cancers. It has recently become established that p53 acts as part of a DNA damage response pathway, therefore, it is possible that this tumour suppressor gene plays an important role in the skin where epidermal keratinocytes are frequently exposed to the damaging effects of UV radiation. Furthermore, it has been reported that viral oncoproteins may contribute to carcinogenesis through inactivation of p53 in anogenital HPV associated malignancies. However, to date there has been no systematic study of the role of p53 in the development of cutaneous lesions in RARs.

### **3.7 AIMS OF STUDY**

The aims of this study therefore, were to determine:

- the prevalence of HPV DNA in benign, premalignant and malignant lesions from RARs and ICPs.
- the prevalence of putative oncogenic HPV types 5, 8, 16 and 18 and the more common cutaneous HPV types 1 and 2, and the common "low risk" genital HPV types 6 and 11, in cutaneous lesions from RARs and ICPs.
- whether the pattern of HPV prevalence in the spectrum of cutaneous neoplasia associated with RARs suggests the stage at which HPV act in the oncogenic process.
- the prevalence of accumulated p53 in pre-malignant and malignant lesions from RARs and ICPs.
- the relationship between positive immunocytochemistry and p53 gene mutations in cutaneous cancers.
- the stage at which p53 acts in the carcinogenic process of RARs and ICPs.
- the relationship between accumulation of p53 and the HPV status of cutaneous lesions from RARs.



**CHAPTER 2:**  
**MATERIALS AND METHODS**

## **CHAPTER 2 : MATERIALS AND METHODS**

### **1: CLINICAL AND HISTOPATHOLOGICAL DATA**

#### **1.1 PATIENT DETAILS**

Two groups of patients were investigated. The first comprised 64 immunosuppressed patients, 61 RARs plus 3 cardiac allograft recipients (mean age 49 years, range 20-70 years), all of whom received transplants between 1965 and 1992 (mean duration of transplant 10.5 years, range 1-26 years). Prior to 1984 patients received immunosuppressive therapy with prednisolone and azathioprine, but since then all new allograft recipients have been treated with prednisolone and cyclosporin A, a few subsequently being switched to azathioprine. All RARs were assessed for their level of sun exposure on the scale of low = indoor occupation, no outdoor leisure activities; moderate = indoor occupation, outdoor leisure activities; high = outdoor occupation and/or more than 3 months living in a tropical climate. The second patient group comprised 83 immunocompetent individuals (mean age 68 years, range 12-94 years) who were referred for treatment of suspected warts or skin malignancies. All IECs/SCCs came from 18 RARs and 48 ICPs. All patients were treated in the Department of Dermatology at the Royal Infirmary of Edinburgh.

#### **1.2 TISSUE COLLECTION**

173 and 89 therapeutic skin biopsies were collected from RARs and ICPs respectively. These included 57 SCCs and 67 IECs. They also included BCCs collected from RARs (7) and ICPs (14) which were not investigated in this study. 6mm biopsies of uninvolved, sun exposed, forearm skin were also obtained from 24 RARs (11 with and 13 without skin tumours elsewhere) and 12 healthy ICPs who volunteered to undergo this procedure. Immediately following excision, each lesion was bisected longitudinally with a sterile blade to minimise the risk of contamination; half was immediately placed in 10% buffered formalin or PLPD (periodate-lysine-paraformaldehyde-dichromate)

(Holgate et al, 1986) and fixed for 24 hours at 4°C before paraffin embedding. Histological assessment (haematoxylin and eosin stained sections) and immunocytochemistry were carried out on paraffin embedded material. The other half was immediately snap frozen in liquid nitrogen and stored at -70°C to await virological investigation and SSCP analysis.

## **PLPD**

### **Part A**

0.1M Lysine

0.1M Periodate

2% Paraformaldehyde

Made up to 50mls in 0.05M phosphate buffer, pH 7.4

### **0.05M phosphate buffer**

12.5g Disodium hydrogen orthophosphate

2g Sodium dihydrogen orthophosphate

2 litres Double distilled water (DDW)

Part A was combined with 5% potassium dichromate in 50mls of distilled water immediately before use.

## **1.3 HISTOPATHOLOGICAL CHARACTERISATION OF LESIONS**

Cutaneous lesions were assessed for standard morphological features suggestive of actinic damage and for degrees of dysplasia progressing to intraepidermal and invasive carcinoma (Blessing et al, 1989). They were designated as viral warts (VWs), actinic and verrucous keratoses (AKs and VKs), intraepidermal carcinomas (IECs) and squamous cell carcinomas (SCCs). VWs showed architectural symmetry, hypergranulosis and koilocytosis. Lesions showing double layered basal budding, basal hypermelanosis, dysplasia and loss of granular layer with superficial parakeratosis were

classified as actinic keratoses. Lesions that showed some features suggestive of HPV infection, but various degrees of basal budding and basal dysplasia, were termed verrucous keratoses. IECs exhibited either full-thickness dysplasia or severe dysplasia in the basal layer. The designation of SCC was confined to lesions where there was evidence of dermal invasion. In some instances, the complex architecture of VK and the variable dysplasia made confirmation of invasion difficult, so the term SCC was used only when dermal invasion was unequivocal (Blessing et al, 1989).

## **2: PREPARATION OF PURIFIED HPV DNA**

### **2.1 TRANSFORMATION OF COMPETENT BACTERIAL CELLS WITH HPV DNA**

Plasmids containing HPV DNA of common cutaneous and EV associated HPV types were received from the original cloning laboratories (Table 2.1). Most of the HPV DNAs were cloned into pBR322 at the tetracyclin resistance site, resulting in an ampicillin resistant plasmid. HPV 19 and 20 were cloned into the pUC9 vector which was also resistant to ampicillin.

Competent HB101 bacteria were prepared by a method based on that of Hanahan (1983). Briefly, a 5ml overnight culture of HB101 E coli cells was used to inoculate 200mls of L-broth which was then incubated for 3 hours at 37°C in an orbital shaker. The bacteria were pelleted by centrifugation at 8000rpm, 4°C for 20min then resuspended in 10mls of TSB and incubated on ice for 30min. The cells, which were now competent, were aliquoted into 200µl aliquots and immediately snap frozen in a dry ice, ethanol bath. In order to transform these bacteria, 100µl of thawed cells were immediately aliquoted into pre-chilled eppendorf tubes. 50-100ng of purified HPV plasmid DNA were then added, mixed gently and incubated on ice for 30min. After heat shock at 42°C for 45sec followed by 2min on ice, 900µl of TSB containing 200mM glucose were added and the sample incubated for 1hour at 37°C, shaking in an

orbital shaker. 100µl and 200µl aliquots of the transformed cells were then plated onto LB-ampicillin plates. This procedure was carried out for each of the HPV types obtained.

### **L-Broth**

10g Bacto-tryptone

10g NaCl

5g Yeast extract

DDW to 1 litre

The L-broth was autoclaved prior to use.

### **TSB**

100mM MgSO<sub>4</sub>

100mM MgCl<sub>2</sub>

1% PEG (3000mw)

0.5% DMSO

L-Broth to 10mls

TSB was sterilised by passage through a 2 micron filter prior to use.

### **LB-Ampicillin plates**

500mls L-broth

7.5g Bacto-agar

50µg/ml Ampicillin

The L-broth plus Bacto-agar were autoclaved then cooled to 55°C and ampicillin added. This solution was poured into approximately 15 petri dishes and left to set.



## **2.2 SMALL SCALE PLASMID PREPARATION**

Five colonies of transformed bacteria were picked from the LB-ampicillin plate and each used to inoculate 10mls of L-broth. Following overnight incubation at 37°C in an orbital shaker (225rpm), 1.5mls of each culture were transferred to an eppendorf tube and microcentrifuged at low speed for 5min (the remainder of the culture was stored at 4°C to await large scale plasmid preparation). The resulting bacterial pellets were resuspended in 100µl of lysis solution, 200µl of alkaline/SDS and 100µl of 3M potassium acetate and incubated for 5min, 5min and 15min respectively at 4°C. The samples were then microfuged at high speed for 5min at 4°C and the supernatant was removed to a clean eppendorf tube. Plasmid DNA was extracted from this solution by the addition of an equal volume of phenol chloroform (x 2) followed by an equal volume of chloroform (the supernatant was removed each time). An isopropanol precipitation was carried out and the plasmid DNA resuspended in 30µl of TE . In order to verify that the plasmid contained HPV DNA, prior to large scale plasmid preparation, each plasmid sample was digested with the enzyme used to clone the particular HPV type (see Table 2.1 for the enzymes used).

### **Lysis Solution**

10mM Tris pH 8

1mM EDTA

100mg/ml Glucose

2mg/ml Lysozyme

### **Alkaline SDS**

1% SDS

0.2M NaOH

## **Potassium Acetate**

3M Potassium acetate

2M Acetic acid

## **TE**

10mM Tris pH 8.0

1mM EDTA pH 8.0

## **2.3 LARGE SCALE PLASMID PREPARATION**

For each HPV type, 500mls of sterile L-broth, containing 50 $\mu$ g/ml of ampicillin, were inoculated with 5mls of transformed bacteria and incubated at 37°C overnight in an orbital shaker. The entire 500ml culture was then transferred to 2 X 250ml polycarbonate centrifuge bottles which were centrifuged at 8000rpm, 4°C for 20min. Following careful removal of the supernatant, the plasmid DNA was recovered using a basic alkaline lysis technique, in one of two ways.

### **Method 1**

The bacterial pellets were resuspended in 10mls of ice cold lysis solution and left at 4°C for 5min. Bacteria were then lysed by the addition of 20mls of alkaline SDS. The solutions were mixed gently by swirling then incubated on ice for a further 5min. Following the addition of 15mls of potassium acetate, the solutions were mixed thoroughly, incubated for 10min on ice, then cleared by centrifugation at 4°C, 8000rpm for 15min. The supernatants were transferred to clean oak ridge tubes and nucleic acid precipitated by the addition of 0.6 volumes of isopropanol at room temperature for 15min. After centrifugation at 8000rpm for 10min at room temperature, the pellet was washed twice with 70% ethanol and air dried. In order to purify further the plasmid DNA, the bacterial pellet was resuspended in 4mls of TE then 4.8g CsCl and 200 $\mu$ l ethidium bromide (10mg/ml) added. This solution was transferred to an ultracentrifuge tube and the plasmid DNA was banded by

centrifugation for 4 hours at 63,000rpm, 20°C on a Beckman ultracentrifuge. The plasmid DNA band was visualised under UV light and removed by inserting a needle into the centrifuge tube and sucking the DNA out into a 3ml syringe. The CsCl was removed by 3 washes with isoamyl alcohol, the plasmid DNA resuspended in 4ml TE (pH 7.5) and precipitated by the addition of 12mls of ethanol at -20°C for 1hour. Subsequent to centrifugation at 10,000rpm for 10min at 4°C, the plasmid DNA pellet was air dried and resuspended in TE to a concentration of 1mg/ml. All solutions were as for small scale plasmid preparation.

## Method 2

The second method utilised the Quiagen kit for maxi-plasmid preparation (Hybaid LTD). The bacterial pellet was resuspended in 10mls of buffer P1, then 10mls of buffer P2 were added and the solution gently mixed. Following incubation for 5min at room temperature, 10mls of buffer P3 were added, mixed immediately, incubated on ice for 20min then centrifuged at 15000 rpm, 4°C, for 30min. In order to ensure a particle free lysate, the resultant supernatant was centrifuged again for a further 10min at 4°C. The supernatant was applied to a Quiagen column 500, which had previously been equilibrated with 10mls of buffer QBT, and allowed to enter the resin by gravity flow. The column was washed twice with 30mls of buffer QC before the purified plasmid DNA was eluted with 15mls of buffer QF. The plasmid DNA was then precipitated in 0.7 volumes of isopropanol at room temperature for 30min followed by centrifugation at 10,000 rpm for 10min at room temperature. The pellet was washed twice in 70% ethanol and dissolved in TE to 1mg/ml. All solutions were supplied in kit form.

## 2.4 VALIDATION OF HPV PLASMID DNA

Five micrograms of purified HPV plasmid DNA were restriction digested as per standard procedure (Sambrook et al, 1989) then electrophoresed through a 0.8% agarose gel (Sigma) containing 0.5µg/ml ethidium bromide. Electrophoresis was

**TABLE 2.1 - SOURCE AND CLONING DATA OF HPV DNA**

HPV TYPE	SOURCE	ENZYME *	FRAGMENT SIZE
HPV1A	P.M Howely (1)	<i>BamHI</i>	1 Fragment - 8Kb
HPV2A	P.M Howely (1)	<i>Bgl II</i>	1 Fragment - 8Kb
HPV3	R.S Ostrow (2)	<i>BamHI</i>	1 Fragment - 8Kb
HPV4	P.M Howely (1)	<i>BamHI</i>	1 Fragment - 8Kb
HPV5/48	H. Pfister (3)	<i>EcoRI</i>	1 Fragment - 1.7Kb
HPV5/49	H. Pfister (3)	<i>EcoRI</i>	1 Fragment - 5.8Kb
HPV8	G.Orth (4)	<i>BamHI</i>	1 Fragment - 8Kb
HPV10	G.Orth (4)	<i>Sall</i>	2 Fragments -6.7, 0.5 Kb
HPV12	G.Orth (4)	<i>HindIII</i>	1 Fragment - 8Kb
HPV13	H. Pfister (5)	<i>BamHI</i>	3 Fragments 5.5, 1.7, 0.6Kb
HPV14	EM. De Villiers (6)	<i>BamHI</i>	1 Fragment - 8Kb
HPV17	G. Orth (6)	<i>BamHI</i>	1 Fragment - 8Kb
HPV19	H. Pfister (6)	<i>BamHI</i>	1 Fragment 8Kb
HPV20	H. Pfister (7)	<i>BamHI</i>	1 Fragment - 8Kb
HPV6b	EM. De Villiers (8)	<i>BamHI</i>	1 Fragment - 8Kb
HPV11	EM. De Villiers (9)	<i>HindIII?</i>	1 Fragment - 8Kb
HPV16	EM. De Villiers (10)	<i>BamHI</i>	1 Fragment - 8Kb
HPV18	EM. De Villiers (11)	<i>EcoRI</i>	1 Fragment - 8Kb

\* Enzyme used to clone the HPV DNA. Apart from HPV19 and 20 which were cloned into pUC9, all the HPV types were cloned into pBR322. (1) Heilman et al, 1980; (2) Ostrow et al, 1983 (3) Pfister et al, 1983a; (4) Kremsdorf et al, 1983; (5) Pfister et al, 1983b; (6) Kremsdorf et al, 1984; (7) Gross et al, 1988; (8) De Villiers et al, 1981; (9) Gissmann et al, 1982; (10) Durst et al, 1983; (11) Boshart et al, 1984.

carried out in TBE, at 50V for approximately 2 hours. A DNA size marker (Kilobase ladder, Gibco) was included on all gels. Restriction fragments were visualised using a UV transilluminator and the image recorded by a camera linked to a Mitsubishi video copy processor. Each purified HPV plasmid DNA was cut with at least three different restriction enzymes, plus combinations of these enzymes, in order to confirm the HPV type.

### **0.8% Agarose**

0.8g Electrophoresis grade agarose

100mls TBE

### **TBE**

0.089M Tris-borate

0.089M Boric acid

0.002M EDTA

### **Loading buffer**

30% Glycerol

0.25% Bromophenol blue

In TBE

## **2.5 PREPARATION OF PURIFIED HPV DNA FOR USE AS PROBES**

Fifteen micrograms of HPV plasmid DNA were digested with the restriction enzyme used to clone that HPV type (Table 2.1) then loaded, along with 10µl of loading buffer, into a large well of a 0.8% low melting temperature agarose gel containing 0.5µg/ml ethidium bromide, then electrophoresed at 50V, 4°C for 2-4 hours. The resulting DNA fragments were visualised and HPV DNA bands extracted from the gel. Every precaution was taken to prevent cross contamination between DNA of different HPV types. HPV DNA was purified using The GeneClean II kit (Stratagene LTD) as per manufacturer's instructions. The solutions used in this procedure were provided in the kit.

The concentrations of purified HPV DNA were measured by aliquoting 5µl of HPV DNA onto ethidium bromide plates alongside 5µl of standard solutions of known



concentration. The concentration of HPV was then estimated by comparing the intensity of UV illumination of HPV DNA to that of the standards.

### **Ethidium Bromide Plates**

1g Agarose (Life Technologies)

10 $\mu$ l Ethidium bromide (10mg/ml)

100mls TBE

5-10mls of agarose solution were poured into plastic petri dishes and left to set.

### **Standards**

Serial dilutions of Kb Ladder (Gibco) were carried out to give final concentrations of 30 $\mu$ g/ml, 15 $\mu$ g/ml, 7.5 $\mu$ g/ml, ~4 $\mu$ g/ml, ~2  $\mu$ g/ml and ~1 $\mu$ g/ml.

## **3: SOUTHERN HYBRIDISATION ANALYSIS**

### **3.1 PREPARATION OF GENOMIC DNA**

A small cube of frozen tissue was added to 700 $\mu$ l of lysis solution in an eppendorf tube and minced using sharp, surgical scissors. 50 $\mu$ l of fresh proteinase K (25mg/ml) were then added and the sample incubated at 37°C in an orbital shaker overnight. Following centrifugation to remove the undigested tissue, the DNA was extracted by a standard phenol chloroform extraction technique (1 x phenol, 1 x phenol/chloroform, 1 x chloroform) (Sambrook et al, 1989). The DNA was then precipitated by the addition of 1 volume of isopropanol followed by incubation at -20°C for 1 hour. Subsequent to centrifugation, the DNA pellet was washed twice with 70% ethanol the pellet was then redissolved overnight in 400 $\mu$ l of TE. 50 $\mu$ l aliquots of genomic DNA were removed from the stock solutions for use in PCR assays. All procedures were carried out using filtered pipette tips to prevent cross contamination of DNA.

## **Lysis Solution**

50mM Tris

50mM EDTA

100mM NaCl

5mM DTT

1% SDS

## **3.2 RADIOACTIVE LABELLING OF HPV DNA**

50-75ng (25ng of each HPV type in mixed probes) of HPV DNA were labelled using a random priming, DNA labelling kit (Megaprime kit, Amersham International) as per manufacturer's instructions. Briefly, 1-10 $\mu$ l of DNA plus 5 $\mu$ l of primer solution and DDW (final volume 50 $\mu$ l) were denatured for 5min at 95-100°C. 10 $\mu$ l of nucleotide mix, 50 $\mu$ Ci (5 $\mu$ l) alpha-<sup>32</sup>P (dCTP) and 2 $\mu$ l of Klenow enzyme were then added and the DNA labelled for 15-20min at 37°C. The buffers and reagents were supplied in kit form.

Labelled DNA was separated from unincorporated, radioactive dNTPs by passage through a Nick Column (Pharmacia) as specified by the manufacturer. Labelled DNA was eluted in 400 $\mu$ l of TE and denatured at 95-100°C prior to use.

## **3.3 DOT BLOT HYBRIDISATION OF HPV PLASMID DNA**

### **3.3.1 Preparation Of Filters**

Dot blot filters containing HPV types 1, 2, 3, 4, 5, 8, 10, 12, 13, 14, 17, 19 and 20 were made for use in initial optimisation experiments. 50pg of purified HPV DNA were denatured (95-100°C for 5min) and spotted onto a charged nylon membrane (Hybond N+, Amersham) which had been pre-soaked in 10xSSC. The filter was then baked for 1 hour at 80°C in a vacuum oven to ensure binding of DNA to the filter.

### **3.3.2 Prehybridisation And Hybridisation**

The filter containing HPV DNA of multiple types was sealed in a plastic hybridisation bag (Hybaid) and 30mls of prehybridisation solution added to one port with a syringe, ensuring that all air bubbles had been removed. Following 6 hours prehybridisation at either 45°C, 55°C or 65°C, denatured labelled probe (95-100°C for 5min), containing one or more HPV types, was added to the hybridisation bag and hybridisation carried out overnight in a shaking water bath at the same temperature as prehybridisation.

### **3.3.3 Washing Conditions**

Hybridisation solution containing the radioactively labelled probe was discarded and unbound radioactivity washed off by running 500mls of 2xSSC over the filter using a Hybaid vacuum pump. 250mls of 2xSSC, 1% SDS were added to the bag which was then incubated at the appropriate temperature (as for hybridisation) for 30min. Some filters were removed from the hybridisation bag at this point, sealed in a plastic bag and exposed to X-ray film (Fugi) (in an x-ray cassette with intensifying screens), either overnight or for 3 days, at -70°C. Some of the filters were washed with a further wash of 0.5xSSC, 1% SDS for 30min and in some cases this was followed by an even more stringent wash of 0.1xSSC, 1% SDS for 30min (all at the same temperature as hybridisation), prior to exposure to X-ray film for 24 hours or 3 days at -70°C. The autoradiographs were developed using standard photographic solutions.

### **3.3.4 Re-Probing**

Bound probe was removed from filters by boiling the filter in 0.1xSSC, 1% SDS for 20min. The filter was then dried on 3mm filter paper (Whatman) to be used again.

## **SSC**

0.15M NaCl

15mM Tri-sodium citrate

Made up in distilled water as a 20X concentrate and diluted in distilled water for use.

## **Prehybridisation solution**

10% Dextran sulphate

1% SDS

6xSSC

100µg/ml denatured and snap cooled salmon sperm DNA

Made up in distilled water and heated to 65°C prior to use.

## **3.4 SOUTHERN HYBRIDISATION ANALYSIS OF GENOMIC DNA**

### **3.4.1 Restriction Digestion Of Genomic DNA**

8-10µg of genomic DNA were digested with the restriction enzyme *BamHI* (NBL) overnight at 37°C. Some samples were also digested with *PstI* (NBL) and *HindIII* (NBL). In all cases, the enzyme was present in a twofold excess and the total volume of the reaction was no less than 30µl. 3µl of loading buffer were added and the samples run on a 20 x 20, 0.8% agarose gel (containing 0.5µg/ml ethidium bromide) at 50mA, room temperature, overnight. Positive (purified DNA of various HPV types diluted in 5µg of restriction digested, placental DNA) and negative (restriction digested placental DNA) controls were included on each gel. To confirm adequate digestion of genomic DNA, the DNA was visualised on a UV transilluminator and the image recorded. The gel bound DNA was then denatured for 30min in 0.5M NaOH, 1.5M Tris prior to transfer to nylon filters.

### **3.4.2 Southern Transfer Of Genomic DNA**

Whatman 3mm filter paper, pre-soaked in 0.5M NaOH, 1.5M Tris, was placed on a plate suspended over a glass dish containing the same alkaline solution, with the ends of this paper serving as a wick to draw up the solution. The gel was then placed on top of this filter paper and a 20 x 20 sheet of charged nylon membrane (Hybond N+, Amersham) placed on top of the gel, followed by 2 sheets of 20 x 20, 3mm filter paper. Bubbles between the gel and membrane were removed prior to the addition of a 3inch stack of absorbent paper towels with a very heavy weight on top to aid capillary movement of the buffer through the gel and membrane. Transfer took place over 16-20 hours before the membrane was removed and neutralised in 3M NaCl, 0.5M Tris (pH 7.4) for 30min.

### **3.4.3 Prehybridisation And Hybridisation**

For filters containing genomic DNA, prehybridisation and hybridisation were carried out as specified for dotblot filters but at a temperature of 55°C. A probe cocktail containing HPV types 3, 8 and 13 was used on all filters, as was a probe cocktail containing HPV types 2 and 12. Each filter was also probed with single HPV types and other combinations of HPV types to a maximum of 4 re-probings. The filters were initially washed with 2XSSC, 1%SDS for 30min at 55°C then exposed to X-ray film for 24 hours. Following development of the autoradiograph, the filter was exposed to X-ray film for a further 3 days. If a high background of radioactivity was present, the filter was washed at 0.5XSSC, 1%SDS for 30 min at 55°C then exposed to X-ray film for a further 1-3 days.

## **4: POLYMERASE CHAIN REACTION**

### **4.1 PRIMER DESIGN**

Oligonucleotide primers, situated in E6 of the HPV genome, were designed from published sequence data (Danos et al, 1982; Fuchs et al, 1986; Zachow et al, 1987;



Hirsch-Behnam, 1990) to detect HPV types 1, 2, 5 and 8 in type specific assays (Table 3.3). The secondary structure, G-C content, specificity (with regards other HPV types) and annealing temperature of all primers were analysed with the aid of the University of Wisconsin (UW) Genetics Computer Group software. Oligonucleotides suitable for primers were synthesised on an Oswel Gene Synthesiser (Dept. of Chemistry, University of Edinburgh, UK) and were HPLC purified. Primers for HPV types 6b, 11, 16 and 18 were received from M.J Arends and had been validated in previous studies (Arends et al, 1991). Consensus primers MY09 and MY11, situated in L1 of the HPV genome and known to amplify genital HPV types, were received from M. Manos (Manos et al, 1989).

#### **4.2 PCR ANALYSIS**

PCR was carried out in a 100µl reaction containing 10µl of pre-prepared x10 reaction buffer (NBL), 200µM of each dNTP (Boehringer), 1.0µM of each primer and 0.5 units of *taq* DNA polymerase (NBL). Purified HPV DNA, diluted in genomic DNA, was used to optimise the PCR assay for each primer pair. The optimal cycle conditions on a Hybaid automated heating block were as follows: one cycle of 94°C for 5min; 30 cycles of 58°C (55°C for HPV1) for 2min, 72°C for 3min and 94°C for 1min; and one cycle of 58°C (55°C for HPV1) for 2min and 72°C for 10min. 30µl of amplified PCR product were run alongside a DNA size marker on a 2% Nusieve:Seakem (3:1) agarose gel (Flowgen Instruments) containing 0.5µg/ml ethidium bromide in TBE buffer. DNA was visualised on a UV transilluminator and the image recorded. Samples were scored as positive when a band of the correct size was visible.

#### **4.3 PCR ANALYSIS OF GENOMIC DNA**

Prior to amplification with HPV primers, each sample was amplified with control *Ki-ras* primers to confirm adequate preservation of DNA. These primers were as follows: p1- GACTGAATATAAACTTGGTGG, p2 - CTCTATTGTTGGATCATATT, and

resulted in a product size of 109bp. A 1µg aliquot of genomic DNA was used as template in a 100µl reaction and positive (1pg and 0.01pg of HPV DNA) and negative (template free and colonic DNA) controls were included in each reaction.

#### **4.4 CONSENSUS PRIMER PCR**

The consensus primer PCR assay was based on that of Lungu et al, (1992). 10pg of HPV types 1, 2, 3, 4, 8, 10, 12, 14, 17, 19, 20, 6 and 18, mixed with 1µg of human placental genomic DNA or 1µg of sample DNA were amplified using the HPV consensus PCR primers described by Manos et al, (1989). In initial studies, PCR reaction conditions were according to the protocol of Manos et al, (1989). 10µl of sample DNA were added to a 100µl reaction mixture containing 50mM KCl, 4mM MgCl<sub>2</sub>, 10mM Tris (pH 8.5), 200uM dNTPs, 1 µM of each PCR primer and 2.5U Taq DNA polymerase (NBL). This reaction mixture was subjected to 35 cycles of 95°C for 1min, 37°C for 1.5min and 72°C for 1.5min. Subsequently, the above buffer was replaced by NBL pre-prepared x10 reaction buffer and the cycle conditions altered to 1 cycle at 95°C for 5min, 30 cycles at 45°C for 1min, 72°C for 2min , 95°C for 1min and 1 cycle at 45°C for 1min, 72°C for 10min.

Following amplification, 30µl of PCR product were electrophoresed as for the standard PCR protocol. To type the product by restriction fragment length polymorphism, the paraffin oil was removed from each HPV positive sample and the remaining product precipitated by the addition of 1 volume of ethanol followed by incubation at -20°C. DNA was pelleted by microcentrifugation, washed in 70% ethanol and resuspended in 1x restriction enzyme buffer containing 10mM Tris HCl (pH 7.9), 10mM MgCl<sub>2</sub> , 50mM NaCl and 1mM DTT. 2µl of a cocktail of enzymes containing 10U each of *HaeIII*, *PstI* and *RsaI* (Boehringer) was then added and the mixture incubated at 37°C for 4-12 hours. The entire reaction was then analysed by gel

electrophoresis as described above. A DNA size marker (Boehringer, marker V) was included on all gels.

#### **4.5 PROCEDURES TO MINIMISE THE RISK OF CONTAMINATION**

Stringent precautions were taken to minimise the risk of contamination of the PCR solution with HPV DNA from unwanted sources. In the preparation of genomic DNA, the scissors used to mince the tissue were stringently cleaned with 1M HCl between cases and pipettes specifically for genomic DNA, with filtered pipette tips, were used during the extraction procedures. Filtered pipette tips were also used when adding genomic DNA to the PCR reaction. Separate laboratories were used for preparation of the PCR reaction, analysis of the PCR products and preparation of genomic DNA. Positive displacement pipettes were used in setting up the PCR reaction which was carried out in a class II biological safety cabinet.

### **5: IMMUNOCYTOCHEMISTRY**

#### **5.1 IMMUNOCYTOCHEMICAL ASSAY FOR THE DETECTION OF ACCUMULATED P53**

3µm sections of PLPD or formalin fixed, paraffin embedded tissue were cut, floated onto glass slides and allowed to dry at 30°C. Sections were dewaxed in xylene, rehydrated in graded alcohols then washed for 5min in TBS. Normal rabbit serum (NRS), diluted 1:1000 with TBS (NRS/TBS), was then applied for 30min followed by incubation with an anti-p53 monoclonal antibody (PAb1801 (DAKO) or MAb Do-7 (Novocastra)), diluted 1:100 with NRS/TBS, for 1 hour (PLPD fixed material) or overnight (formalin fixed material). Bound antibody was detected by treatment with biotinylated rabbit antibody to mouse immunoglobulin (1:400 NRS/TBS) for 10min followed by incubation with avidin-biotin complex linked to horseradish peroxidase (Dako) for 30min. After TBS washing, bound antibody was visualised with diaminobenzidine (1mg/ml in 0.2M Tris pH 7.6, 0.03% hydrogen peroxide) for 2 min

followed by a tap water wash, a light haematoxylin counterstain and dehydration in graded alcohols and xylene, prior to mounting under coverslips.

### **Tris Buffered Saline**

145mM NaCl

20mM Tris pH 7.6

## **5.2 ASSESSMENT OF EXTENT OF IMMUNOPOSITIVITY**

Each section was scored by two independent observers and the extent of staining recorded on the following graded scale: 1=<10%, 2=10-50%, 3=>50% of cells in a lesion showing positive nuclear staining. Sections were scored as positive when immune precipitate was visible in > 10% of cells in the lesion i.e., grades 2 and 3 only. The significance of sparse nuclear p53 staining is presently unknown, therefore, lesions with grade 1 score were considered to be negative. The histological localisation of stabilised p53 within each lesion was also noted.

## **6: SINGLE STRAND CONFORMATIONAL POLYMORPHISM AND SEQUENCING TO DETECT P53 MUTATIONS**

### **6.1 PRC OF EXONS 5 TO 8 OF P53 FOR SSCP ANALYSIS**

The polymerase chain reaction was performed on 0.1-1µg of tumour genomic DNA (and in most instances, paired normal DNA), in a 100µl reaction with reagents as specified in Section 4.2 of the materials and methods and primers specific for exons 5, 6, 7 and 8 of the p53 gene (Table 2.2). PCR was performed in an automated thermocycler (Hybaid) with the following temperature profile: 1 cycle of 94°C for 5min, 30 cycles of 94°C for 1min, 58°C for 1min, 72°C for 1min and 1 cycle of 72°C for 10min.

**6.2 SSCP ANALYSIS OF PCR PRODUCTS**

The amplified products were extracted once with 24:1 chloroform/isoamylalcohol to remove any mineral oil. 5-10µl of purified products were then denatured in 80uM NaOH, 10uM EDTA, at 48°C for 5min, 10µl of sequencing stop solution was then added and the whole sample loaded onto a 5% glycerol, 0.5x MDE Hydrolink gel (Hoefer Scientific). The gel was run in 1X TBE on the SE600 PAGE apparatus (Hoefer Scientific) at 25°C, 20W for 2-3 hours. Bands were visualised by a silver stain (Biorad) as per manufactures instructions. The gel was dried onto 3mm paper and laminated.

**TABLE 2.2 PRIMERS USED IN SSCP ANALYSIS OF EXONS 5 TO 8 OF p53 AND SEQUENCING OF EXON 7**

p53 Exon	Primer	Sequence
5	p1	TTCCTCTTCCTACAGTAGTC
5	p2	CGATGGTGGAGCAGCTGGG
6	p1	CCTCACGTATTGCTCTTAGG
6	p2	CTGAGGTCTGGTTTGCAACT
7	p1	TGTGTTTCTCCTAGGTTGG
7	p2	GTCAGGAGCCACTTGCCA
8	p1	TCCTATCCTGAGTAGTGGT
8	p2	CGAGGTAAGCAAGCAGGA
890	P1	GAGAGAAGCTTGCCACAGGTCTCCCCAAGGCGAA
891	p2	AGAGAGGGATCCACTTTCCACTTGATAAGAGGTCCCAAG
5-6	p1	AGGAGGTGCTTACACATGTT

**6.3 CLONING OF PCR PRODUCTS FOR SEQUENCING EXON 7 MUTATIONS**

**6.3.1 PCR Of Genomic DNA**

Genomic DNA from samples found to have an exon 7 mutation by SSCP analysis were amplified using primers 890 and 891 (Table 2.2) that amplify exons 7-9 of the p53 gene. Standard reaction conditions, as previously specified for HPV DNA, were applied (see Section 4.2 of Materials and Methods). The PCR reaction consisted of 30



cycles of 96°C for 1.3min, 53°C for 1.6min and 71°C for 2.5min. On completion of the reaction, the entire sample was electrophoresed through a 1% low melting temperature gel. Following extraction of the amplified band, the DNA was purified from agarose using the Geneclean II kit (as specified by the manufacturer, Stratech LTD) and the concentration measured using ethidium bromide plates (see Section 2.5 of Materials and Methods). The purified product was then restriction digested by adding 2µl of *Bam*HI enzyme, 2µl of *Hind*III enzyme, 2µl *Bam*HI enzyme buffer and 4µl of water then incubating at 37°C for 1hour. 5µl of digested product were run on an 1% agarose gel to test for adequate digestion and the remaining digested product was purified once more using Magic Cleanup Columns (Promega) as specified by the manufacturer. 50ng of pUC18 were also restriction digested using *Bam*HI and *Hind*III and purified with Magic Cleanup Columns. The concentrations of restriction digested insert and vector were estimated using ethidium bromide plates.

### **6.3.2 Ligation**

Approximately 10ng of vector and 5ng of insert were ligated in a reaction containing 1mg/ml bovine serum albumin, 1mM ATP, 1 x ligase buffer and 1µl T4 DNA ligase (New England Biolabs). The reaction was incubated overnight at 16°C and used to transform competent DH5α E.coli as specified for HPV DNA (see Section 2.1). 5-10 transformed colonies were pooled and cultured overnight in 10mls of L broth. Miniprep DNA was prepared from these cultures using a Magi Mini-prep kit (Promega) as per manufacture's instructions.

#### **10 X Ligase Buffer**

500mM Tris pH 7.6

100mM MgCl<sub>2</sub>

100mM Dithiothreitol

### **6.3.3 Sequencing**

5ng of plasmid DNA were initially mixed with 10ng of primer DNA (the upstream exon 7 primer used for SSCP analysis) and denatured by the addition of 1µl of NaOH at 37°C for 10min. The mixture was then neutralised with 1µl 1M HCl and 2µl 5x sequencing buffer added and incubated for a further 5min at 37°C. Sequencing was then carried out using the dideoxy chain termination method with the Sequenase 2.0 kit (United States Biochemical) as per manufacturer's instructions. All the solutions were supplied in kit form.

### **6.3.4 Polyacrylamide Gel Electrophoresis**

A 0.25mm thick denaturing, polyacrylamide gel was poured between the glass plates of a Gibco BRL sequencing apparatus and allowed to polymerise. The gel was then pre-run at 40W until it had reached 50°C. 2µl of denatured (75°C for 2min) sequencing reaction (4 reactions per sample) were loaded onto the gel and the gel run at 40-50W until the bromophenol blue dye had run through the gel once. Following separation of the plates, the gel was fixed in 12% methanol, 10% acetic acid for 2 X 10min. Once the final fix was removed, the gel was adhered to a piece of Watmann 3mm filter paper, vacuum dried at 80°C for 1hour then exposed to X ray film (Kodak) overnight at -70°C. Following development using standard photographic solutions, the sequence was read by eye.

### **6% Urea Acrylamide Gel**

6% Acrylamide:bisacrylamide (39:1)

1 X TBE

1µl 25% Ammonium persulphate per ml of gel

1µl TEMED per ml of gel

#### **6.4 USE OF DYNABEADS FOR SEQUENCING EXON 5 MUTATIONS**

Sequencing was performed by this method on samples found to contain exon 5 mutations by SSCP analysis.

##### **6.4.1 PCR Of Genomic DNA**

PCR of exons 5 to 6 was carried out using a biotinylated upstream primer (p1 in Table 2.2) and the exon 6 reverse primer (p2) as for SSCP analysis. The reaction components were as previously specified (see Section 4.2 of Materials and Methods) and the cycling conditions were as for SSCP analysis (see above).

##### **6.4.2 Preparation Of Single Stranded DNA**

The two strands of PCR amplified products were separated using DYNABEADS (Dynal). 20µl of DYNABEADS per sample were prewashed in 60µl of 1x B+W buffer. The beads were then placed next to a magnet and the wash buffer removed. Following resuspension in 100µl of B+W buffer, the beads were added to 100µl of amplified product and mixed periodically for 15-20min. The samples were then placed next to a magnet, the supernatant removed and the sample washed once in B+W buffer. The supernatant was removed once again followed by 2 5min incubations with 20µl of 1M NaOH. The samples were washed once in 200µl of B+W buffer and once in 200µl TE then resuspended in 5µl DDW. 5µl of single stranded DNA attached to DYNABEADS were used in each sequencing reaction.

##### **B+W Buffer**

10mM Tris pH 7.5

1mM EDTA

2M NaCl

### **6.4.3 Sequencing And Electrophoresis**

Sequencing and gel electrophoresis were carried out as for cloned DNA using the Sequenase 2.0 kit.

## **CHAPTER 3:** **RESULTS**



## **CHAPTER 3: RESULTS**

### **1: CLINICAL AND PATHOLOGICAL FEATURES**

#### **1.1 CLINICAL FEATURES**

Renal allograft recipients residing in the Edinburgh area are routinely monitored for the presence of cutaneous lesions including VWs, keratoses and skin malignancies. During the period 1989-1993, 173 cutaneous biopsies were collected from 19 female and 33 male RARs (Table 3.1). 6mm punch biopsies of non-involved, sun-exposed forearm skin were also obtained from a further 11 male and 2 female patients. All IEC/SCC came from 18 RARs whose details are given in tables 3.2A and B. Seven patients (patients 16, 20, 23, 34, 54, 26 and 64), all with graft lives of 10 years or more, exhibited multiple dysplastic and malignant skin neoplasms over the 4 year collection period (Tables 3.2A and B and appendix 1). However, 9 of the 15 patients with graft lives of 15 years or more, exhibited no, or very few, cutaneous lesions (Table 3.2A). Conversely, 1 IEC and 1 SCC were collected from a patient with a graft life of only 2 years (Table 3.2B). Face, hands, scalp (in bald men) and forearms were the most common sites for VW and skin cancer development although, a small number of IECs and SCCs were removed from non sun-exposed body sites. Although the majority of patients with IECs/SCCs had moderate to high sun exposure, some patients with multiple cutaneous SCCs had low sun exposure whilst other patients with graft lives of >15 years and high sun exposure, exhibited no skin cancers (Table 3.2A).

103 lesions from 83 ICPs were also obtained during the period of this study (Table 3.1). BCCs are far more common than SCCs in ICPs and therefore, SCCs from ICPs were difficult to obtain for this study. Overall, cutaneous lesions from ICPs were derived from an elderly, chronically sun-exposed population with lesions on sun-

exposed body sites. The 48 ICPs exhibiting IECs/SCCs were on average, over 20 years older (mean age 75 years) than RARs with comparable lesions (mean age 54 years).

**1.2 HISTOPATHOLOGICAL FEATURES**

Histological examination of VWs, keratoses, IECs and invasive SCCs from RARs revealed a spectrum of change from a typical viral wart to an invasive SCC (Figure 3.1). The majority of VWs from RARs exhibited mild to moderate dysplasia as well as viral features, such as symmetry of lesion, papilliferous architecture, irregular patterns of vascularity and focal koilocytic change (Figure 3.1A). Likewise, some degree of dysplasia was observed in all verrucous keratoses from RARs (Figure 3.1B). In fact, in 8 of these lesions, this dysplasia was full thickness and almost amounted to IEC (Figure 3.1C). The combination of viral features and severe dysplasia was also observed in several SCCs which showed viral architecture and cytological features at one margin and typical features of an SCC at the other (Figure 3.1D). However, in general, the viral features diminished as dysplastic and invasive aspects emerged.

**TABLE 3.1:- CUTANEOUS LESIONS FROM RAR AND ICP**

PATIENTS	HISTOLOGICAL TYPE OF LESION							
	VW	AK	VK	IEC	SCC	BCC	MISC.	US
<b>RAR</b>	28	28	20	35	41	7	14	24
<b>ICP</b>	8	15	3	32	17	14	2	12

RAR = renal allograft recipient; ICP = immunocompetent patient; VW = viral wart; AK = actinic keratoses; VK = verrucous keratoses; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma; BCC = basal cell carcinoma; MISC. = miscellaneous lesions (including 5 seborrheic warts and 4 keratoacanthomas); US = uninvolved, sun-exposed forearm skin.

**TABLE 3.2:- DISTRIBUTION OF LESIONS PER PATIENT****A: PATIENTS With a Graft Life Of  $\geq 15$  Years**

PATIENT DETAILS						CUTANEOUS LESIONS					
Code	Age/Sex (m/f)	Graft Life (yrs)	Sun	Aza/ CyA	High Risk	VW	K	IEC	SCC	US	MISC
1	44 m	15	Mod	Aza		-	-	-	-	1	-
3	39 m	18	Low	Aza		1	-	-	-	1	-
12	55 f	15	Low	Aza		1	6	-	1	-	-
16	49 m	17	Mod	Aza	+	-	4	3	6	1	1
20	36 f	21	Low	Aza	+	1	2	4	9	-	-
21	60 m	23	High	Aza		-	-	-	-	1	-
23	56 f	23	Low	Aza	+	4	4	7	1	-	1
34	44 m	26	High	Aza	+	1	1	3	8	-	-
41	45 m	21	Low	Aza		-	-	-	-	1	-
46	42 m	16	High	Aza		-	-	-	-	1	-
49	46 f	25	Low	Aza		-	1	-	-	-	-
54	52 m	15	High	Aza	+	-	5	2	3	1	1
57	48 m	15	Mod	Aza		-	1	-	-	-	-
60	43 f	20	Low	Aza		1	-	-	-	-	-
61	58 f	15	Low	CyA		-	1	-	-	-	-

**B: Patients Exhibiting IEC/SCC With A Graft Life Of <15 Years**

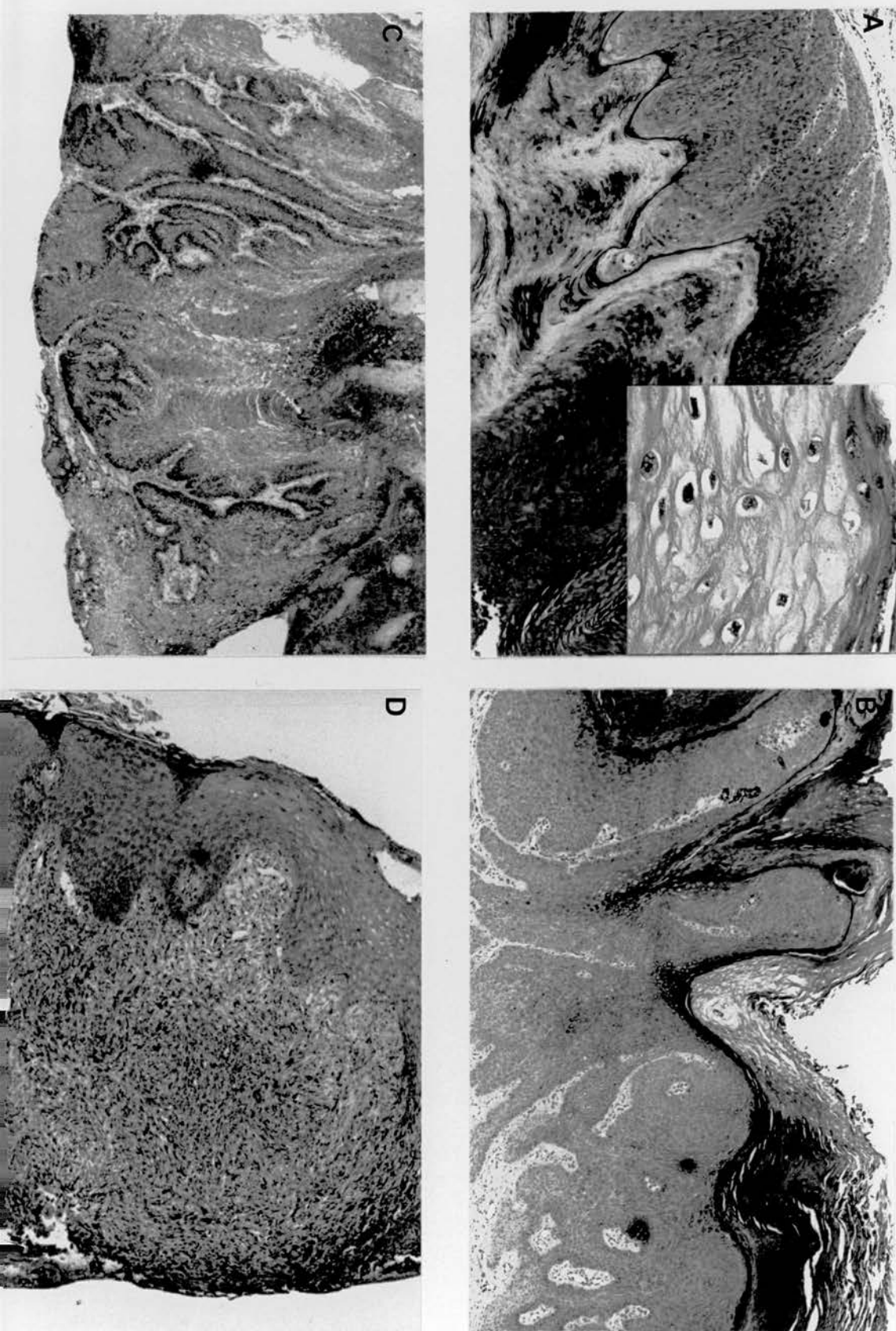
PATIENT DETAILS						CUTANEOUS LESIONS					
Code	Age/Sex (m/f)	Graft Life (yrs)	Sun	Aza/ CyA	High Risk	VW	K	IEC	SCC	US	MISC
6	55 m	2	Mod	Aza		-	-	1	1	1	-
8	56 m	6	Mod	CyA		-	2	1	-	-	-
13	58 m	5	High	CyA		-	-	1	-	-	-
14	56 m	4	Low	CyA		-	-	1	-	1	-
26	52 m	10	High	Aza	+	3	5	4	7	-	-
27	66 m	8	High	CyA		-	1	2	-	1	-
39	59 m	9 C	uk	CyA		-	-	-	1	-	-
40	59 m	13	High	Aza		-	2	1	-	1	-
53	33m	6	High	CyA		1	-	1	-	-	-
58	76 m	10	High	Aza		-	-	-	1	-	-
59	69 m	1	Low	CyA		-	-	1	-	-	1
64	57m	12	High	Aza	+	-	1	3	3	1	-

Lesions collected at the Royal Infirmary of Edinburgh, Department of Dermatology, during the period 1989-1993.

Sun exposure: low = very little, mod = outdoor leisure activities, high = outdoor occupation or more than 3 months in a tropical climate. Aza = azathioprine; CyA = cyclosporin A; m/f = male/female; VW = viral wart; K = keratoses; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma; US = uninvolved, sun-exposed forearm skin; MISC = miscellaneous lesions (including BCCs); C = cardiac allograft recipient; uk = unknown.

**Figure 3.1:-** (A) Viral wart exhibiting papilliferous architecture. Inset shows cell vacuolation (koilocytic change) and cytoplasmic inclusions at high power. (B) Verrucous keratosis with the topography of a viral wart but lacking the cytological features. There is some irregularity of the basal tongues. (C) Verrucous keratosis with widespread dysplasia amounting to intraepidermal carcinoma. (D) Invasive squamous cell carcinoma arising from a surface exophytic verrucous keratosis (haematoxylin and eosin).

FIGURE 3.1:- HISTOLOGICAL FEATURES OF CUTANEOUS NEOPLASMS OF RENAL ALLOGRAFT RECIPIENTS





## **2: HPV IN CUTANEOUS NEOPLASIA OF RENAL ALLOGRAFT RECIPIENTS**

### **2.1 DESIGN AND OPTIMISATION OF HPV DETECTION ASSAYS**

#### **2.1.1 Validation Of HPV Probes**

Prior to use in Southern hybridisation and PCR assays, all HPV containing plasmid DNAs were digested with multiple restriction enzymes in order to confirm that they were of the specified HPV type. The restriction digest patterns of all HPV types used in this study were as published. Figure 3.2 demonstrates the validation of HPV 5 and 8 plasmid DNA. HPV 10 was received as two plasmids, one containing the 6.7Kb and the other the 0.5Kb *Bam*HI fragments. Only the 6.7Kb fragment was used in optimisation assays and as a DNA probe. The two *Eco*RI fragments of HPV 5C had also been cloned separately (HPV5/48 and 5/9 in Figure 3.2A). Control HPV 5 DNA and HPV 5 DNA probes contained a mixture of both these fragments. The three *Bam*HI fragments of HPV 13 were previously cloned by partial digestion into a single pBR322 vector. In this case, DNA probes contained a mixture of the two larger fragments (5.5Kb and 1.7Kb), purified separately. For all other HPV types, the full HPV genome was used in optimisation assays, as controls for Southern hybridisation analysis and as DNA probes.

#### **2.1.2 Optimisation Of The Southern Hybridisation Assay**

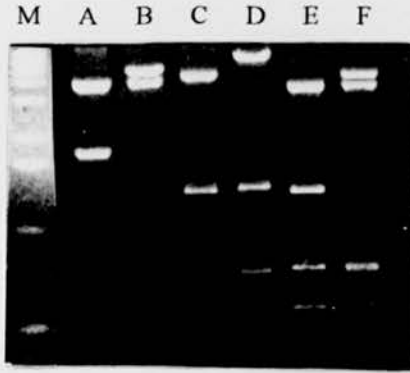
Dot blot filters containing purified HPV DNA of types 1, 2, 3, 4, 5, 8, 10, 12, 13, 14, 17, 19 and 20, were prepared and used to optimise a Southern hybridisation assay in which multiple HPV types could be detected using a single cocktail of HPV probes. Initial experiments indicated that at low hybridisation ( $T_m$  -35°C) and washing ( $T_m$  -40°C) stringency, a single HPV probe, containing either HPV type 8 or 12, is able to detect HPV types 5, 8, 12, 14, 17, 19 and 20. Similarly, a DNA probe of HPV type 2 or 3 was found to cross hybridise with HPV types 2, 3 and 10, and an HPV 13

probe with HPV types 1, 2, 3, 10, 13, 19 and 20, under the same hybridisation and washing stringencies. Subsequently, it was found that by using a probe cocktail of HPV types 3, 8 and 13 at a low hybridisation ( $T_m$ - 35°C) and washing ( $T_m$  -40°C) stringency, it was possible to detect HPV types 1, 2, 3, 5, 8, 10, 12, 13, 14, 17, 19 and 20 (Figure 3.3 A). A probe cocktail containing HPV types 2, 4 and 12 was also found to cross hybridise with multiple HPV types, including HPV type 4 which was not consistently detected in the other assay. Neither probe cocktail hybridised to control human genomic DNA or pBR322 under these conditions of stringency (Figure 3.3A). The results obtained from dot blot filters were confirmed, with identical HPV type specificity, by performing Southern hybridisation analysis on control genomic DNA (placental DNA) spiked with purified HPV DNA (Figure 3.3B). HPV 6,11,16 and 18 were not tested in these assays, although they are known to hybridise to HPV 13 (de Villiers, 1989).

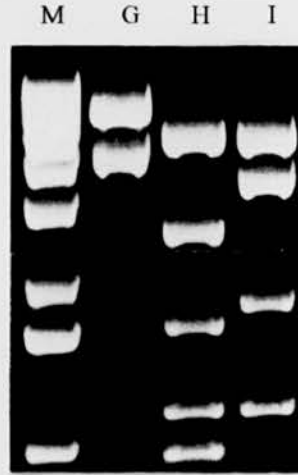
The sensitivity of the Southern hybridisation assay was assessed using serially diluted, purified HPV 16 DNA mixed with a known concentration of control genomic (placental) DNA. These preliminary investigations demonstrated that Southern hybridisation analysis, using similar hybridisation and washing conditions and an HPV 16 probe radioactively labelled to a specific activity of  $4 \times 10^6$  c.p.m/ ml, could detect 5pg of viral DNA in a background of 10ug of genomic DNA following an overnight exposure to X-ray film (Figure 3.4A). This detection level is equivalent to 0.2 copies of HPV DNA per cell. Southern analysis, using mixed HPV probes to detect multiple HPV types, was sensitive to 50pg of viral DNA in a background of 10ug of genomic DNA, which is equivalent to 2 copies of HPV DNA per cell.

**FIGURE 3.2:- VALIDATION OF HPV TYPE 5 AND 8 PLASMID DNA**

HPV5



HPV8

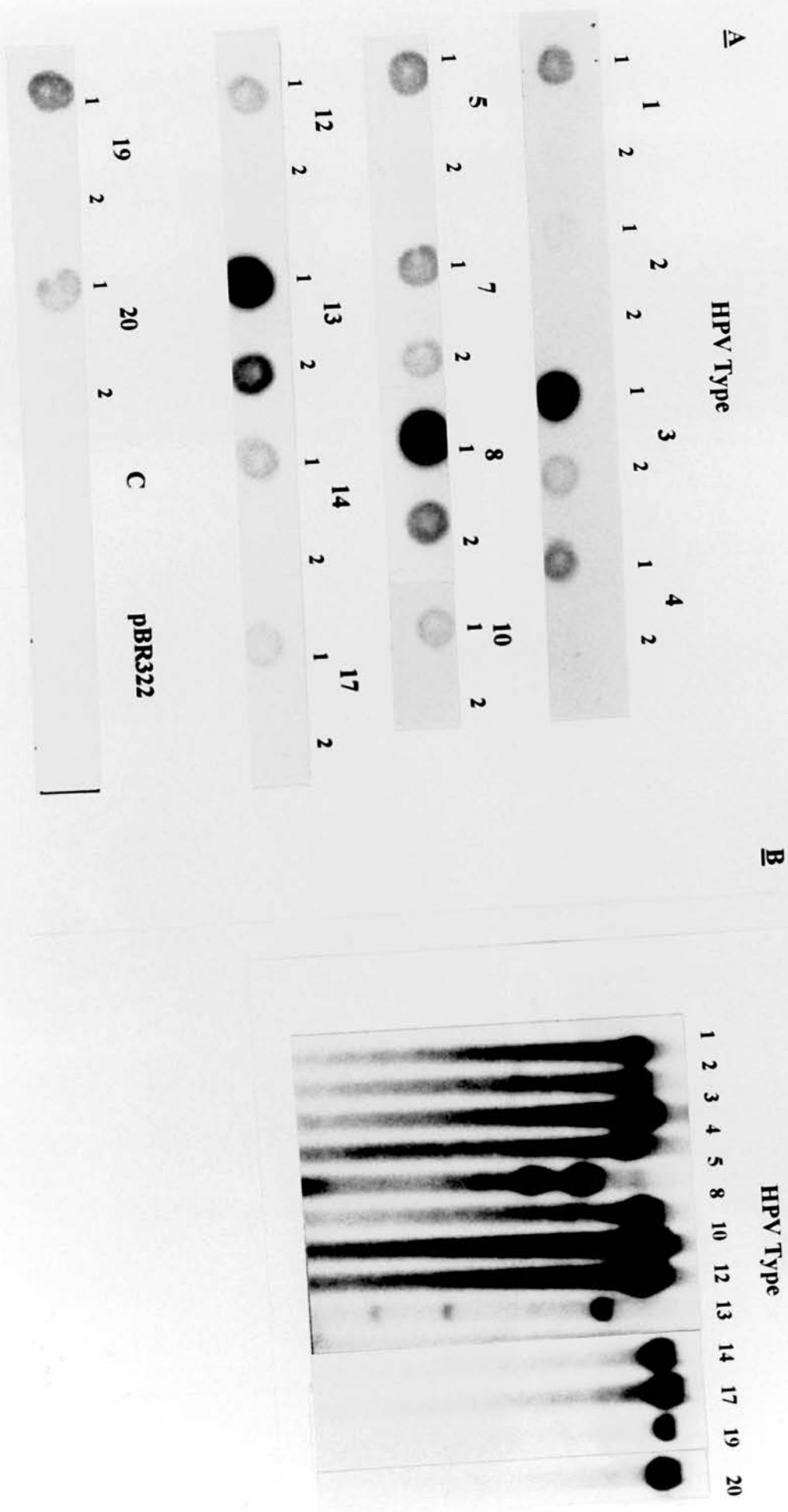


Track	Plasmid	Enzyme	Predicted Size Of Fragments*	Actual Size Of Fragments*
A	HPV5/48	<i>EcoRI</i>	4, 1.7	4, 1.7
B	HPV5/9	<i>EcoRI</i>	4, 5.8	4, 5-6
C	HPV5/48	<i>PstI</i>	4.7, 1.2	4-5, 1.3
D	HPV5/9	<i>PstI</i>	8.7, 1.2, 0.75	9-11, 1.4, 0.8
E	HPV5/48	<i>EcoRI</i> + <i>PstI</i>	3.4, 1.3, 0.7, 0.5	3.5, 1.3, 0.8, 0.6
F	HPV5/9	<i>EcoRI</i> + <i>PstI</i>	5.1, 3.4, 0.7, 0.75, 0.5	5-6, 3-4, 0.75, 0.7, 0.5
G	HPV8	<i>BamHI</i>	8.0, 4.0	4.0, 8.0
H	HPV8	<i>BamHI</i> + <i>PvuII</i>	5.2, 2.8, 1.8, 1.2, 1.0	5.0, 2.5, 1.7, 1.3, 1.0
I	HPV8	<i>PvuII</i>	5.2, 3.5, 1.9, 1.22	5.0, 3-4, 1.8, 1.2

M = 1Kb Ladder (Gibco) size marker; HPV5/48 = 1.7Kb *EcoRI* fragment of HPV5C cloned into pBR322; HPV5/9 = 5.8Kb *EcoRI* fragment of HPV5C cloned into pBR322; HPV8 = full length HPV 8 cloned into pBR322. \* = Length of restriction fragments in Kb.

**Figure 3.3:-** (A) Autoradiograph of dot blot filter containing HPV types 1, 2, 3, 4, 5, 7, 8, 10, 12, 13, 14, 17, 19 and 20 probed with a probe cocktail of HPV types 3+8+13 at low hybridisation (T<sub>m</sub>-35°C) and washing (T<sub>m</sub>-40°C) stringency. 1 = 50pg of purified HPV DNA; 2 = 5pg of purified HPV DNA; C = control human genomic (placental) DNA; pBR322 = 50pg of purified pBR322 plasmid DNA. (B) Autoradiograph of Southern hybridisation analysis performed on 100pg of purified HPV DNA (of types 1 to 20) mixed with 10µg of *Bam*HI restricted, human genomic (placental) DNA, hybridised with a probe cocktail of HPV types 3+8+13 at low hybridisation (T<sub>m</sub>-35°C) and washing (T<sub>m</sub>-40°C) stringency.

**FIGURE 3.3:- OPTIMISATION OF THE SOUTHERN HYBRIDISATION ASSAY FOR THE DETECTION OF MULTIPLE HPV TYPES**





### **2.1.3 Design of PCR Primers For HPV Types 1, 2, 5 And 8**

Several factors were taken into account when designing primers for the HPV detection assay. In anogenital cancers, viral integration has been shown to disrupt all but the URR and the E6 and E7 ORFs. In addition, E6 from HPV types 5 and 8 is known to be oncogenic in transformation assays (Watts et al, 1984; Iftner et al, 1988; Fuchs & Pfister, 1990). Therefore, the E6 ORF was chosen as the target sequence for primer design. In order to design absolutely type specific PCR primers for HPV types 5 and 8, the nucleotide sequences of which are over 75% homologous, the University of Wisconsin (UW) computer program GAP was used to identify sequences unique to each HPV type. This program allows the determination of the exact extent of base mismatching of the most similar HPV types. Using this program, the published nucleotide sequences within the Genbank/EMBL databases of HPV types 5 and 8 were compared to each other and to those of HPV 1 and 2. Regions with a high degree of mismatching at either end of the E6 ORF were selected for the design of unique PCR primers for each HPV type. Within these regions, sequences suitable for efficient primers were identified on the basis of several other criteria, including GC content, predicted secondary structure and 3' complementarity, following guidelines set out by Saiki (1989). Primers of 25-30bp with an even distribution of GC to AT nucleotides were chosen to allow the use of stringent annealing temperatures, further ensuring type specificity. The sequences chosen for use as oligonucleotide primers are shown in Table 3.3.

### **2.1.4 Optimisation Of PCR Assays For HPV Types 1, 2, 5 And 8**

The PCR reaction conditions for all primer pairs were optimised by performing PCR on serially diluted, purified HPV DNA mixed with a known concentration of genomic (placental) DNA, over a range of annealing temperatures. Primers for HPV types 2, 5 and 8 were found to specifically amplify HPV DNA at an optimal annealing temperature of 58°C whereas the optimal annealing temperature for HPV 1 primers

was 55°C. Denaturing, annealing and extension times of 1min, 2min and 3min respectively were more efficient than equivalent times of 30sec, 1min and 1min. For all HPV types, these optimal reaction conditions allowed detection of 0.01pg of HPV DNA in a background of 1ug of genomic DNA which is equivalent to  $4 \times 10^{-3}$  copies of HPV genome per cell (Figure 3.4B). Each set of primers was also tested against a panel of cloned HPV types 1, 2, 3, 4, 5, 8, 10, 12, 13, 14, 17, 19 and 20, and found to be absolutely type specific.

**TABLE 3.3:- HPV PRIMER SEQUENCES USED TO DETECT HPV TYPES 1, 2, 5 AND 8**

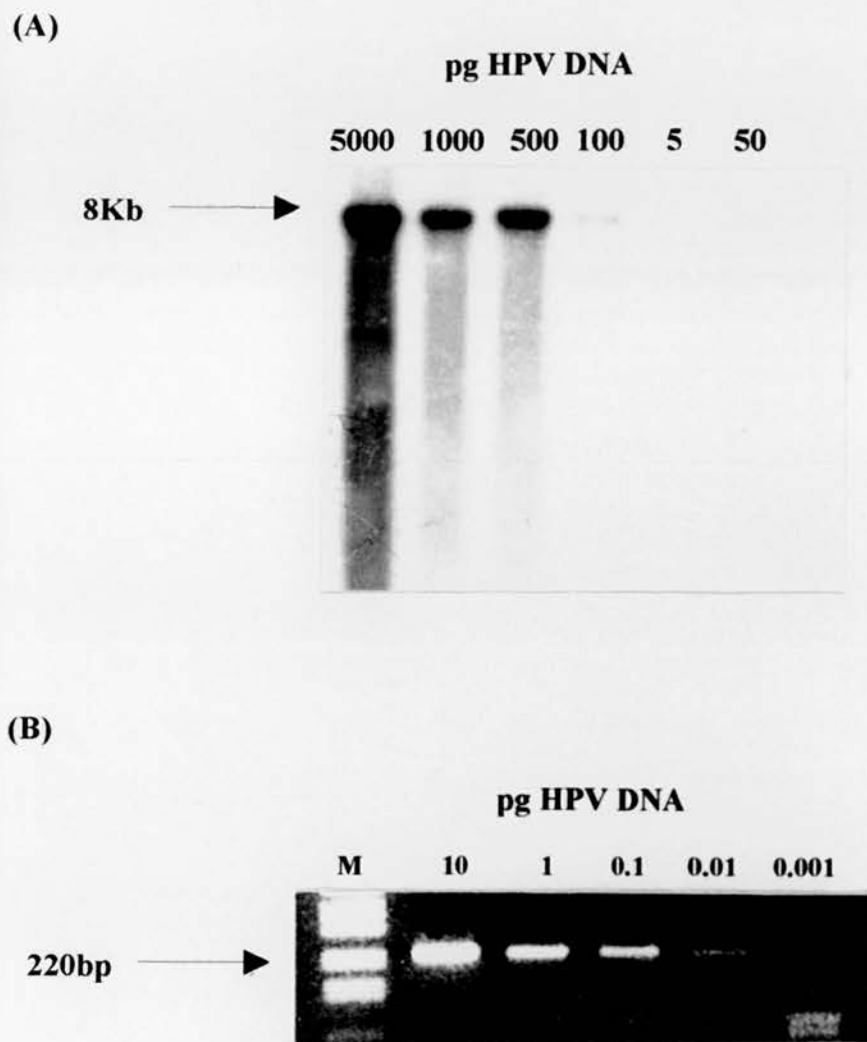
HPV	Primer	Sequence	Position*	Product
1 1	p1 p2	AGTCTTATGAGGTACCGGAAATAGAAG ATGCACTCTTTCTCCGTTTGACACAACCTC	383-409 519-490	137bp
2 2	p1 p2	ATGGTTTGGAGCTAGAGGATTTGCG AACTAGTAATGCCTCCTTCTCCTCC	159-183 463-439	305bp
5 5	p1 p2	CTCTAATACCAAATTCTGTGGCGT GAGGAACGCCTGGAAGGGAATCTG	616-640 894-870	279bp
8 8	p1 p2	CGGGCAGGACAAGGCTTCATATTTAGACAC ACAACAACGACAACACGCAGTAACAAC	201-230 420-394	220bp

\* = Nucleotide position in HPV genome defined by EMBL/Genebank database

**2.2 HPV PREVALENCE IN CUTANEOUS NEOPLASIA FROM RAR AND ICP**

A total of 125 skin biopsies from RARs, including 60 IEC and SCC specimens from 16 patients, together with 71 specimens from ICPs were analysed by Southern hybridisation using the mixed probe cocktails 3, 8 and 13 and 2, 4 and 12 (as described above), to screen for the presence of HPV DNA. As expected, detection of HPV DNA was greatest in VWs (50%), but 23% of keratoses, 29% of IECs and 41% of SCCs from RARs contained HPV DNA (Table 3.4 and Figure 3.5). In both

**FIGURE 3.4:- SENSITIVITY OF HPV DETECTION ASSAYS**



**(A)** Autoradiograph of Southern analysis performed on serially diluted, purified HPV 16 DNA mixed with  $10\mu\text{g}$  of *Bam*HI digested genomic (placental) DNA. The filter was probed with a full length HPV 16 probe at high hybridisation ( $T_m$ - $25^\circ\text{C}$ ) and washing ( $T_m$ - $30^\circ\text{C}$ ) stringency and exposed to X ray film overnight. Under these conditions of stringency, 5pg of viral DNA could be detected in a background of  $10\mu\text{g}$  of genomic DNA which is equivalent to  $0.2$  copies of HPV per cell. **(B)** Ethidium bromide stained gel of serially diluted, purified HPV 8 DNA, mixed with  $1\mu\text{g}$  of genomic DNA, amplified using HPV 8 primers. Using the conditions specified in the text (see 2.3.1), 0.01pg of HPV 8 DNA could be detected in a background of  $1\mu\text{g}$  of genomic DNA which is equivalent to  $4 \times 10^{-3}$  copies of HPV genome per cell. Identical results were obtained for the primers designed to amplify HPV types 1, 2 and 5 (not shown). M = 1Kb molecular weight marker (Gibco).

populations, only one specimen (out of 21 for RARs and 12 for ICPs) from normal skin was positive for HPV DNA. Within the RAR group, this rate of positivity was significantly less than that of SCCs (41%;  $p < 0.01$ ), IECs (29%;  $p < 0.05$ ), and VWs (50%;  $p < 0.001$ ) using a  $\chi^2$  test. Except for 6 viral warts, lesions from ICPs showed lower HPV DNA prevalence than those from RARs (Table 3.4). Statistical comparison of the results for each histological category between RARs and ICPs revealed that IEC/RAR differed significantly ( $p < 0.05$  by  $\chi^2$  test) from IEC/ICP. Statistical significance was not reached for the comparison of SCCs due to the small number of SCCs from ICPs analysed.

**TABLE 3.4 - HPV DNA PREVALENCE DETECTED BY SOUTHERN HYBRIDISATION ANALYSIS**

Patient Group	Number (%) lesions positive				
	VW	K	IEC	SCC	US
RAR	7/14 (50)	7/30 (23)	9/31 (29)	12/29 (41)	1/21 (5)
ICP	6/6 (100)	1/13 (8)	1/25 (4)	3/15 (20)	1/12 (8)

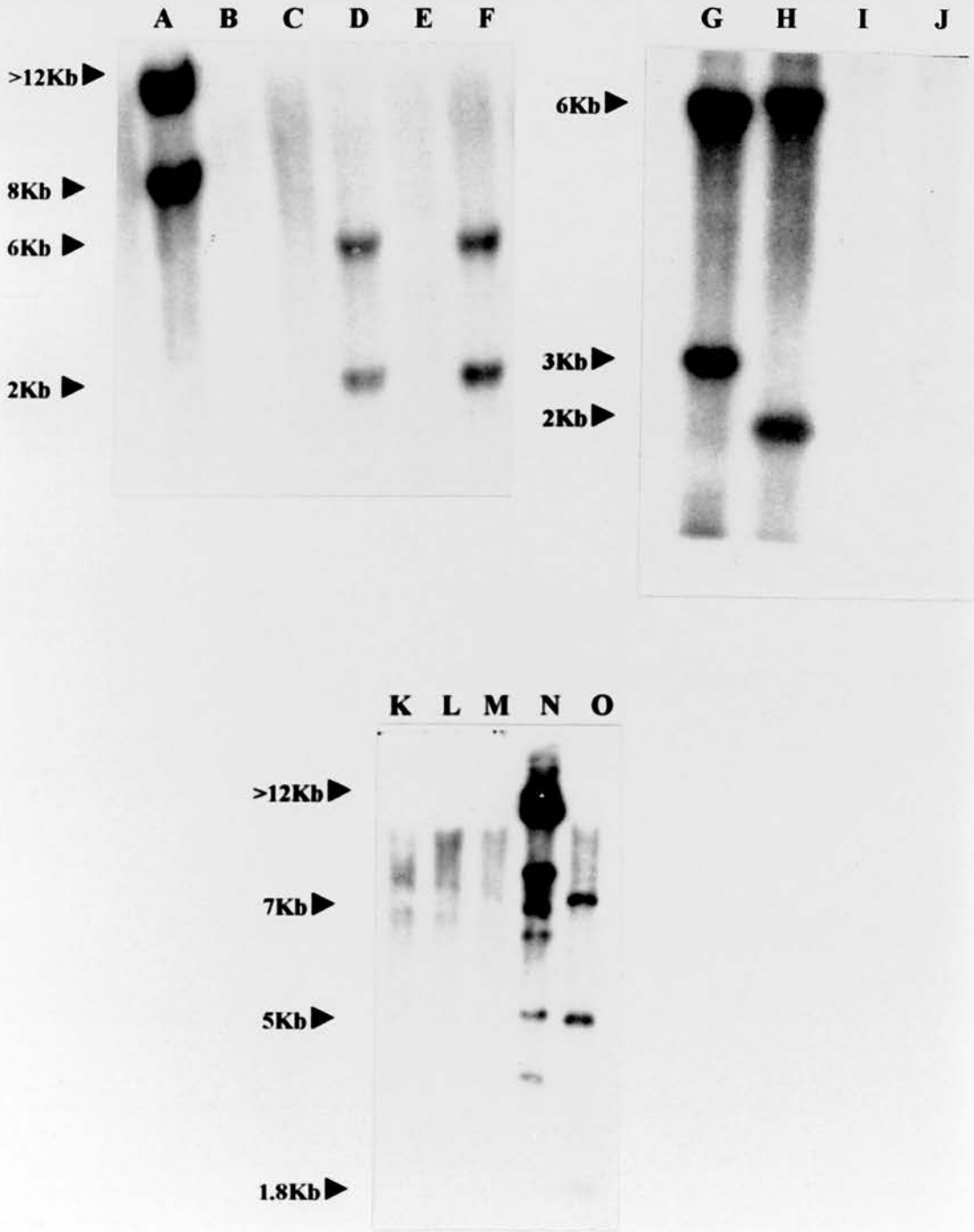
RAR = renal allograft recipient; ICP = immunocompetent patient; VW = viral wart; K = keratosis; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma; US = uninvolved, sun-exposed skin.  
 $\chi^2$  tests revealed significant differences of  $p < 0.001$  for comparisons of VW/RAR with US/RAR,  $p < 0.05$  for comparison of IEC/RAR with US/RAR,  $p < 0.01$  for SCC/RAR versus US/RAR, and  $p < 0.05$  for IEC/RAR versus IEC/ICP.

**2.3 HPV TYPES AND PHYSICAL STATUS OF HPV DNA IN CUTANEOUS NEOPLASIA FROM RAR**

Some of the cases found to be HPV positive by the initial screen for HPV DNA were further analysed by high stringency Southern hybridisation (Tm -50°C for hybridisation and Tm -55°C for washing) with single HPV probes and by Southern hybridisation

**Figure 3.5:-** Autoradiographs showing HPV positive and negative cutaneous lesions. All specimens were from immunosuppressed patients and the size markers are indicated. A probe cocktail of HPV types 3+8+13 was used with *Bam*HI digested genomic (sample) DNA. Track (A) is an HPV positive SCC (sample 77) from patient 34; tracks (G) (sample 68) and (N) (sample 18) are HPV positive dysplastic keratoses; tracks (D) (sample 82), (F) (sample 88), (H) (sample 70) and (O) (sample 17) are HPV positive viral warts. See Appendix 1 for patient codes of samples. Track A shows evidence of HPV genome integration within a DNA fragment greater than 12Kb in size. Track N shows multiple restriction fragments, the sum of which are greater than 8Kb. This banding pattern was reproducible suggesting that viral integration may also have occurred in this sample. Tracks D, F, G and H show episomal HPV genomes cleaved into two fragments of approximately 2Kb and 6Kb (see Table 3.5 for details of the typing of these samples). Tracks (I), (J) and (M) are HPV negative SCC; (C) and (K) are HPV negative IEC; (B) (this sample was HPV 16 positive by PCR) and (E) are HPV negative keratoses. (L) is an SCC which was negative when probed with the HPV 3+8+13 probe cocktail but was HPV positive when probed with a probe containing HPV types 2 and 12. This sample was also HPV 2 positive by type specific PCR.

**FIGURE 3.5:- LOW STRINGENCY SCREEN FOR HPV DNA BY SOUTHERN  
HYBRIDISATION ANALYSIS**





using the restriction enzymes *PstI* and *HindIII*, in an attempt to determine more precisely the type of HPV present (Table 3.5, Figure 3.6). Although some of the restriction patterns obtained were similar to those of published HPV types, none were identical to those published for HPV types 1 to 57. Overall, multiple different common cutaneous and EV-associated HPV types appeared to be present in both dysplastic VWs and pre-malignant and malignant skin tumours from RARs. In one IEC, only a single 3-3.5Kb band was present when the lesion was digested with both *BamHI* and *PstI*, while in other cases, the *HindIII* and *PstI* digests resulted in multiple bands, the sum of which were greater than 8Kb. Due to the apparently small copy numbers of HPV genomes within lesions from ICPs, no attempt was made to further type the HPV detected by the initial screen of these lesions.

Restriction pattern analysis identified complex patterns of HPV DNA bands, that could not be explained by single episomal forms of HPV types 1 to 57 (for which restriction enzyme patterns have been well characterised), in 2 VWs, an actinic keratosis and an SCC from 4 separate RARs. When digested with the single cut enzyme *BamHI*, these cases gave either multiple restriction fragments (sample 18, Figure 3.5), the sum of which was greater than 8Kb but dissimilar to the size of multiple episomes or, a high molecular weight band of >12Kb (samples 46, 77 and 78, Table 3.5, Figure 3.5). Similarly, *PstI* digestion resulted in multiple high and low molecular weight bands, the sum of which was greater than 8Kb, in these samples (Figure 3.6A and D). These banding patterns were reproducible, excluding incomplete digestion. Possible explanations for these complex banding patterns include integration of HPV genomes, infection with two different HPV types (both episomal or episomal and integrated), intra-episomal DNA rearrangements such as URR duplications, or a combination of these. These possibilities cannot be reliably distinguished by the available data, although, band sizes of >12Kb favour the possibility of viral genome integration.

**TABLE 3.5 - TYPE AND PHYSICAL STATUS OF HPV DNA IN CUTANEOUS NEOPLASIA FROM RAR**

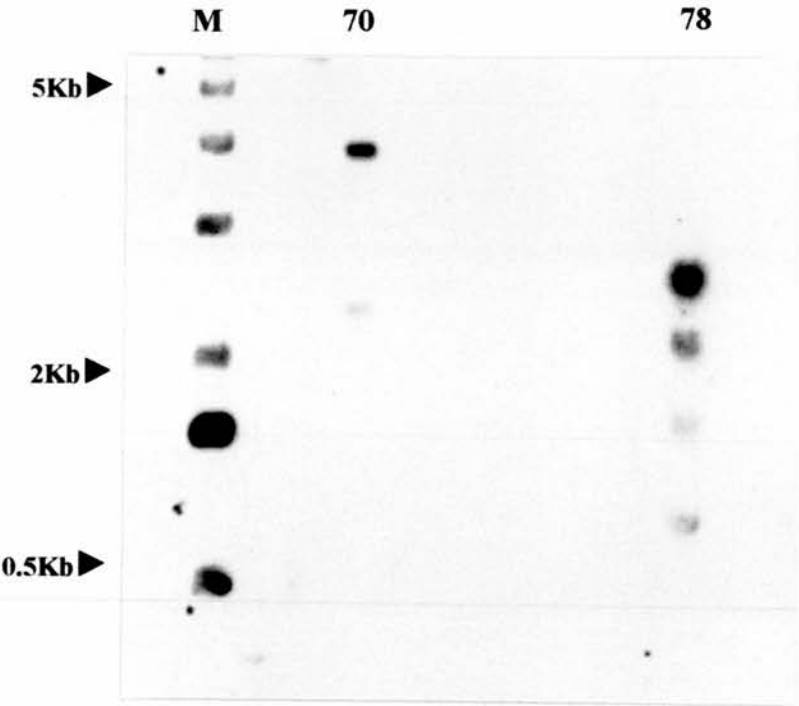
SAMPLE	LESION	ENZYME	HPV PROBE #	BAND SIZES (KB)	TYPE*/ INTEGRATION
17 <sup>(1)</sup>	VW	BamHI Hind III PstI	3+8+13 3 3	7.0, 5.0, 1.8, 0.8 8.0, 9-10 1.3, 6.0	13 or 20
46	VW	BamHI PstI	8 2+12	>12 >12	3A or int/mul
70	VW	BamHI HindIII PstI	3+8+13 3 3	1.5, 6.5 uncut 4.0, 2.5	2A@
78	VW	BamHI PstI	3+8+13 13	>12, 8.0 2.8, 2.5, 1.6, 1.3	int/mul
88	VW	BamHI PstI	3+8+13 13	2-3, 6.0 6.0, 1.0, 0.8	10
18 <sup>(2)</sup>	AK	BamHI  HindIII PstI	3+8+13  3+8+13 3+8+13	>12, 8.0, 5.0, 4.0, 2.0, 1.5 2.6, 7.0 5.5, 5.0, 6.0	int/mul
68	VK	BamHI HindIII PstI	3+8+13 3 3	2-3, 6.0 uncut 6.0, 1.0, 0.8	10
24 <sup>(2)</sup>	IEC	BamHI HindIII PstI	3+8+13 3+8+13 3+8+13	8 2.7, 1.9, 1.6 2.0, 2.4, 2.8	12
5 <sup>(1)</sup>	IEC	BamHI PstI	3+8+13 3+8+13	3.0 3.5	uk
133	IEC	BamHI PstI	3+8+13 2+12	6.0-8.0 2.5, 5.5	3C
21 <sup>(2)</sup>	SCC	BamHI PstI	3+8+13 3+8+13	6.0 2.8, 3.5, 4.0	uk
49 <sup>(1)</sup>	SCC	BamHI PstI	3+8+13 8+2+12	>12, 8.0 8.0	3A
67 <sup>(3)</sup>	SCC	BamHI PstI	8+2 8+2+12	8.0 >12	uk
77 <sup>(3)</sup>	SCC	BamHI PstI	8+2 14**	>12, 8.0 5.0, 6.0	14 or int/mul

# = All Southern analysis was carried out at low hybridisation (T<sub>m</sub> -35°C) and washing (T<sub>m</sub> -40°C) stringency; \* = the most closely related HPV type to that of the sample, as determined by restriction fragment length analysis. None of the samples had restriction fragment banding patterns identical to HPV types 1 to 57; int/mul = HPV integrated or sample infected by multiple HPV types; @ = this sample was HPV 2 positive by PCR; \*\* = this sample hybridised to an HPV 14 probe at high hybridisation (T<sub>m</sub> -45°C) and washing (T<sub>m</sub> -48°C) stringency. VW=Viral wart; AK=actinic keratoses; VK=verrucous keratoses; IEC=intraepidermal carcinoma; SCC=squamous cell carcinoma. (1), (2) and (3) = patients exhibiting multiple typed samples (see Appendix 1 for patient codes of samples)

**Figure 3.6:-** Southern hybridisation autoradiographs showing DNA from cutaneous lesions, digested with a variety of restriction enzymes and probed with HPV probes at varying stringencies. The molecular weights of fragments are shown. M = 1Kb Ladder (Gibco) size marker. (A); Two HPV positive viral warts. In both samples, the DNA was digested with the restriction enzyme *PstI* and the resulting filter probed with HPV 13 at medium stringency. Sample 78 gave a high molecular weight band (>12Kb) when digested with BamHI providing evidence for viral integration into the host cell genome. Sample 70 was HPV 2 positive by type specific PCR. (B); DNA from a dysplastic keratosis (sample 68) digested with *PstI* (track 1) and *HindIII* (track 2) and probed with an HPV 3 probe at high stringency. The sizes of the resulting restriction fragments were similar to those published for HPV 10. (C); DNA from a viral wart (sample 88) digested with *PstI* and probed with HPV 2+12 at low stringency. This sample also has a restriction pattern similar to HPV 10. (D); DNA from a dysplastic keratosis (sample 18) digested with *PstI* (track 1) and *HindIII* (track 2) and probed with HPV 3+8+13 at low stringency. This sample appears to contain integrated HPV DNA. See Table 3.5 for details of the typing of HPV in these samples.

**FIGURE 3.6:- TYPING OF HPV IN CUTANEOUS NEOPLASMS BY SOUTHERN HYBRIDISATION ANALYSIS**

**(A)**



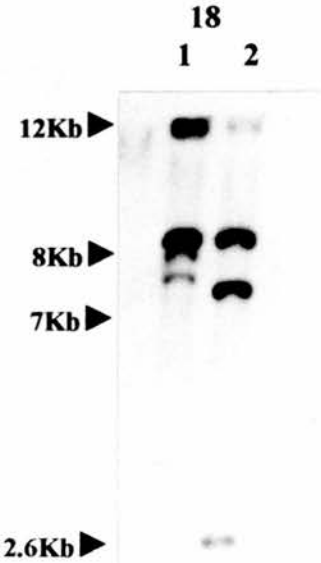
**(B)**



**(C)**



**(D)**



## **2.4 PREVALENCE OF SPECIFIC HPV TYPES IN CUTANEOUS LESIONS FROM RAR AND ICP, AS DETERMINED BY TYPE SPECIFIC PCR ASSAYS**

A total of 139 specimens from RARs and 81 from ICPs were analysed by type specific PCR for HPV types 1, 2, 5 and 8 (Tables 3.6A and B, Figure 3.7). In each sample, *c-Ki-ras* sequences could be detected with appropriate *ras* primers. Relatively few lesions were positive for HPV DNA compared with the results from Southern hybridisation analysis. In particular, HPV 5 DNA was only present in a small number of benign and premalignant lesions from RARs and ICPs and was not detected in any SCCs. HPV 8 DNA was found in only one SCC from an ICP. Interestingly, HPV 5 DNA was present in two uninvolved skins from RARs whilst HPV 1 and 2 were detected in both premalignant and malignant lesions from RARs and ICPs (Tables 3.6A and B). Overall, there was no dominant HPV type in any of the histological categories and the distribution of types was broadly similar for immunosuppressed and immunocompetent patients.

One hundred and sixteen and 73 specimens from RARs and ICPs respectively, were also tested for the common genital HPV types 6, 11, 16 and 18 (Tables 3.6A and B). Genital HPV types were detected in 9 cutaneous specimens from RARs compared to none from ICPs. There was no dominant HPV type in any of the histological categories. Two IECs, from 2 separate RARs, were repeatedly found to have 2 highly specific bands of 600 and 700bp, when amplified with HPV 16 primers (Figure 3.7C). This is compared to the expected product size of 346bp. Four VWs from RARs contained more than one HPV type; 5 and 2, 5 and 6, 5 and 11 and 2 and 11. Rigorous anti-contamination procedures were followed throughout (Arends et al, 1991) and there was no evidence to suggest that any of these positive results were due to contamination from other sources.

**TABLE 3.6: HPV TYPE PREVALENCE BY TYPE-SPECIFIC PCR**

**A: Renal Allograft Recipients**

Histological Type of Lesion	HPV Type and Number Positive Lesions										
	Number Examined	1	2	5	8		Number Examined	6	11	16	18
VW	20	0	4	3	0		15	1	2	0	0
K	33	2	2	1	0		25	0	0	1	0
IEC	33	0	2	1	0		30	0	0	2	0
SCC	31	1	2	0	0		26	1	0	1	0
US	22	0	0	2	0		20	0	0	1	0

**B: Immunocompetent Patients**

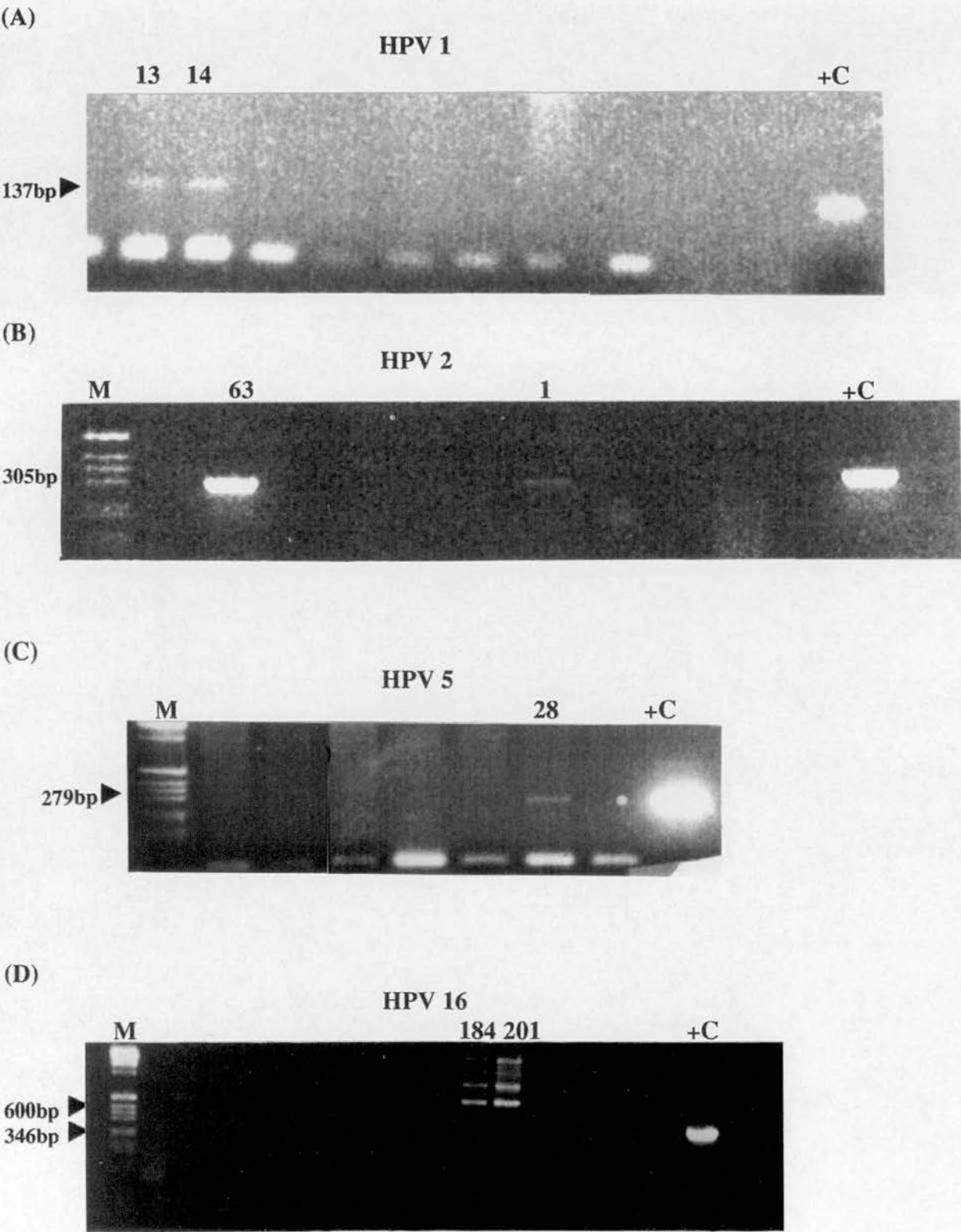
Histological Type of Lesion	HPV Type and Number of Positive Lesions										
	Number Examined	1	2	5	8		Number Examined	6	11	16	18
VW	7	1	1	0	0		6	0	0	0	0
K	15	0	0	2	0		14	0	0	0	0
IEC	31	2	1	2	0		27	0	0	0	0
SCC	16	0	1	0	1		14	0	0	0	0
US	12	0	1	0	0		12	0	0	0	0

VW = viral wart; K = keratoses; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma; US = uninvolved, sun-exposed skin



**Figure 3.7:-** Ethidium bromide stained agarose gels showing HPV positive and negative cutaneous neoplasms by type specific PCR. **(A)**; Two HPV 1 positive dysplastic keratoses (samples 13 and 14) from RAR patient 54; +C = 10pg of purified HPV 1 plasmid DNA. **(B)**; An HPV 2 positive viral wart (sample 63) and squamous cell carcinoma (sample 1) from two separate allograft recipients; +C = 10pg of purified HPV 2 plasmid DNA. **(C)**; An HPV 5 positive intraepidermal carcinoma (sample 28) from renal allograft recipient 20; +C = 100pg of purified HPV 5 plasmid DNA. **(D)**; Two intraepidermal carcinomas from two separate renal allograft recipients showing specific bands of 600bp and 700bp. This is compared to the expected PCR product size of 346bp (+C track); +C = 10pg of purified HPV 16 plasmid DNA. See Table 3.8 for patient details of these samples. M = molecular weight marker (1Kb ladder, Gibco).

**FIGURE 3.7:- THE USE OF TYPE SPECIFIC PCR TO DETECT HPV IN CUTANEOUS NEOPLASMS**



## **2.5 CONSENSUS PRIMER PCR**

A consensus primer PCR assay was used in an attempt to develop a rapid and sensitive method for screening for multiple HPV types and also as a means of determining the HPV type present in lesions found to be positive by the low stringency Southern hybridisation screen. The assay involved amplification of sample DNA with consensus PCR primers as described by Manos et al (1989), followed by restriction enzyme digestion of the products with a cocktail of enzymes described by Lungu et al (1992). The Manos consensus primers were designed primarily for the detection of genital-associated HPV types, therefore, reaction conditions were initially optimised to allow the detection and identification of common cutaneous and EV-associated HPV types. A primer annealing temperature of 45°C was found to amplify HPV types 1, 2, 3, 4, 5, 8, 10, 12, 17, 20, 6 and 18 in the presence of genomic DNA. However, while the PCR reaction was highly sensitive for the control HPV types 6 and 18, the quantity of amplified product was often very poor for the skin-associated HPV types (Figure 3.8A). HPV 14 and 19 were not amplifiable using these consensus primers. When digested with the combination of enzymes described by Lungu et al (1992), the RFLPs of the EV-associated HPV types were found to be very similar (Table 3.7). The enzyme *MnII*, which cuts HPV 5 and 8 at different sites, was therefore added to the cocktail but the restriction digest patterns of HPV 8, 12 17 and 20 were still almost indistinguishable. The RFLPs observed when the amplified products of the control HPV types 6 and 18 were digested using the above enzyme cocktail were very nearly as expected (Table 3.7).

Of 17 cutaneous lesions (mostly VWs and keratoses) known to contain HPV DNA by low stringency Southern hybridisation and type specific PCR assays, only 8 exhibited HPV DNA by consensus primer PCR; 4 VWs, 3 keratoses and 1 IEC (Table 3.7 and Figure 3.8B). However, consensus primer PCR did reveal HPV DNA in 2 of the 25

**TABLE 3.7 :- CONSENSUS PRIMER PCR/RFLP ANALYSIS ASSAY APPLIED TO CUTANEOUS HPV TYPES AND CUTANEOUS SAMPLES FROM RAR**

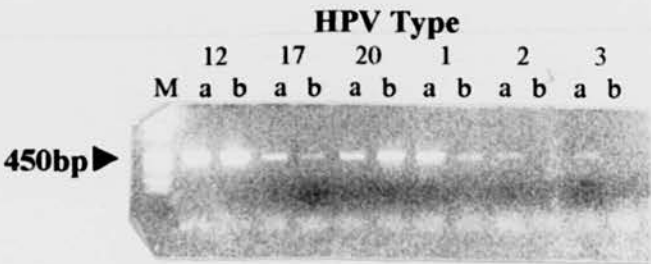
Sample	Fragment sizes (bp) #	Total (bp)**
HPV1	350, 100	450
HPV4	210, 230	440
HPV10	220, 230	450
HPV12	320, 134	450
HPV17	320, 140	460
HPV20	360, 120	480
HPV6	124, 3x80, 57, 30*	450
HPV18	134, 100, 2x80, 30 *	420
17 (VW)	320, 140	460
43 (VW)	100x2, 201, 50	450
110 (VW)	270, 100, 50	420
140 (VW)	450 + 330 + 100	450 + 430
18 (K)	250, 110, 70	430
68 (K)	210, 230	440
13 (K)	>12Kb	-
Gt1 (VIN I)	450, 370, 70	450 + 440
Gt2 (Anal cancer)	210, 130, 70x2	480
Gt3 (CIN II)	300, 220, 70x2	660
Gt4 (Vulval wart)	370, 70	440

# = Samples digested with the enzyme cocktail *HaeIII* + *PstI* + *RsaI*; \* = the published RFLPs are 122, 78, 73, 71, 67, 37 bp for HPV 6 and 134, 107, 85, 73, 38, 18 bp for HPV 18 (Lungu et al, 1992); \*\* = the product size of the Manos L1 consensus primers is 450bp. VW = viral wart; K= keratoses; VIN = vulval intraepithelial neoplasia; CIN = cervical intraepithelial neoplasia. GT = genital tract lesion. See Appendix 1 for the association between sample numbers with patient codes.

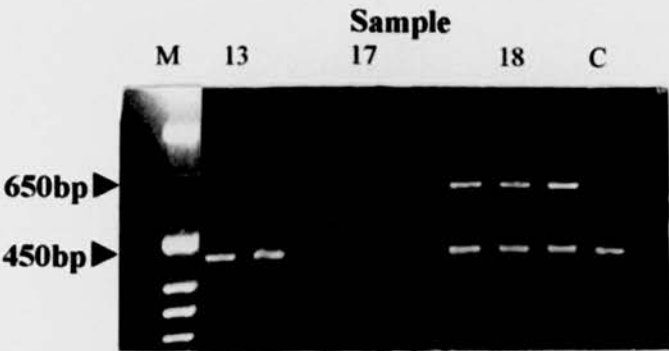
skin specimens investigated (an SCC and a VW) in which no HPV DNA had previously been detected. Two distinct amplification products of 450 and 600bp were repeatedly observed when sample T18 (a keratosis) was amplified using the Manos consensus primers (Figure 3.8B). This sample was believed to contain integrated HPV DNA by Southern hybridisation analysis (Table 3.5). Amplification product was obtained for all 4 genital tract lesions assayed (Figure 3.8B). With one exception, the

**FIGURE 3.8:- CONSENSUS PRIMER PCR APPLIED TO CUTANEOUS HPV PLASMIDS AND CUTANEOUS NEOPLASMS FROM RARS**

(A)



(B)



(A) Ethidium bromide stained agarose gel showing HPV plasmid DNA amplified using the Manos consensus PCR primers that were designed to detect multiple genital associated HPV types. **a** = template of 1ng of purified HPV DNA; **b** = template of 1ng of purified HPV DNA mixed with 1ug of genomic (placental) DNA. (B) Ethidium bromide stained agarose gel showing cutaneous lesions from RARs amplified using the Manos consensus PCR primers. **13** = a dysplastic keratosis that was previously HPV 1 positive by type specific PCR; **17** = a viral wart that was positive by low stringency Southern hybridisation and showed restriction digest patterns similar to HPV 13 or 20 (Table 3.5 and Figure 3.5); **18** = an actinic keratosis that was positive by low stringency Southern hybridisation and was believed to contain integrated viral DNA (Table 3.5 and Figure 3.6); **C** = control sample of a genital lesion known to contain HPV 16 DNA; **M** = molecular weight marker (1Kb Ladder, Gibco).

RFLPs of the HPV types in the above samples, including the 4 genital tract lesions obtained from 2 female RARs, were dissimilar to those published by Lungu et al, (1992) and to those of the HPV types investigated in this study by type specific PCR and Southern hybridisation analysis (Table 3.7). However, the RFLP of sample 68, which had previously been shown to contain HPV 10 DNA by Southern hybridisation analysis, was very similar to the RFLP of HPV 10 in this assay (Tables 3.5 and 3.7). Overall, the consensus primer PCR/RFLP analysis assay worked well on plasmid DNA of genital HPV types but the results obtained when it was applied to cloned HPV of common cutaneous and EV-associated HPV types, and to skin samples known to contain HPV DNA, were difficult to interpret.

## **2.6 CORRELATION OF HPV DNA DETECTION BY SOUTHERN HYBRIDISATION AND TYPE SPECIFIC PCR**

Twenty eight specimens of keratoses, IECs and SCCs from RARs exhibited HPV DNA by low stringency Southern hybridisation analysis, however, only 3 of these were HPV positive by type specific PCR (Table 3.8). Likewise, of the 17 specimens of keratoses, IECs and SCCs from RARs that were HPV DNA positive by type specific PCR, only 3 (out of 16 analysed) were positive by the Southern hybridisation screen (Table 3.8). A combination of both detection assays resulted in 10/14(71%) VWs, 13/30(43%) keratoses, 13/31(42%) IECs, 15/29(52%) SCCs and 3/21(14%) uninvolved skin from RARs containing HPV DNA (Table 3.9). The combined results for ICPs gave HPV prevalences of 6/6 (100%) for VWs, 2/13 (15%) for keratoses, 5/25 (20%) for IECs, 4/15 (27%) for SCCs, and 1/12 (8%) for US (Table 3.9). No statistically significant differences by the  $\chi^2$  test were found comparing HPV prevalence in each histological group between ICPs and RARs. However, SCCs ( $p<0.002$ ) and IECs ( $p=0.05$ ) from RARs showed a significantly higher HPV prevalence than uninvolved skin from RARs. Overall, HPV DNA was detected at a



**TABLE 3.8:- CLINICO-PATHOLOGICAL DETAILS OF HPV POSITIVE LESIONS FROM RENAL ALLOGRAFT RECIPIENTS**

PATIENT			HISTOLOGY	SITE	HPV TYPE	
Number	Age/Sex	Graft Life (yrs)			Southern *	PCR
8	56m	6	AK (D+++)	Face	pos uk	neg
			IEC	Up/back	pos uk	neg
16+	49m	17	SCC	Scalp	pos	2
			SCC	Ear	pos uk	neg
			IEC	Hand	neg	16 #
64+	57m	12	AK (D+)	Finger	pos **	neg
			SCC	Hand	pos uk	neg
			IEC	Ear	pos 12	neg
18	55m	6 (c)	AK (D++)	D. hand	pos uk	neg
			VK	D. hand	pos 10	neg
20+	36f	21	IEC	Presternal	pos	5
			VK	Thigh	ND	5
			SCC	Neck	neg	16
			SCC	Chest	neg	2
			SCC	Chest	pos uk	neg
			IEC	L. thumb	pos uk	neg
			IEC	D. hand	pos 3C	neg
			SCC	Forearm	neg	6
23+	56f	23	IEC	Finger	pos uk	neg
			SCC	Temple	pos uk	neg
			IEC	Thumb	neg	16 #
26+	52m	10	VK (D+)	Neck	pos uk	neg
			IEC	Up. back	pos uk	neg
			SCC	Neck	pos uk	neg
			SCC	Hand	pos 3A	neg
			SCC	Shoulder	pos uk	neg
			SCC	Shoulder	pos	1
34+	44m	26	IEC	Scalp	neg	2
			SCC	Forearm	pos uk	neg
			SCC	Neck	pos uk **	neg
			IEC	Scalp	pos uk	neg
			SCC	Scalp	pos uk	neg
40	59m	13	IEC	Scalp	neg	2
50	62m	8	AK (D++)	Forearm	neg	16
			VK (D+)	Forearm	pos uk	neg
54 +	52m	15	VK (D+)	Forearm	neg	1
			AK	Forearm	neg	1
			VK	Forearm	pos uk	neg
			VK (D+++)	Thigh	neg	2
			VK (D+)	Neck	neg	5
59	69m	1	IEC	D. hand	pos uk	neg
61	58f	15	VK (D+)	Thumb	neg	2

\* = Details of the typing of these samples are given in Table 3.5; + = high risk subset of patients; \*\* = these samples appeared to be integrated (Tables 3.5 and 3.7, Figure 3.8). AK = Actinic keratosis; D+ = mild dysplasia; D++ = moderate dysplasia; D+++ = severe dysplasia; SCC = squamous cell carcinoma; IEC = intraepidermal carcinoma; VK = verrucous keratosis. D. hand = dorsum of the hand; Up back = upper back. ND = not done; uk = unknown; neg = negative. # = These two lesions gave unusual bands when amplified with HPV 16 (see Section 2.4). See Appendix 1. for sample numbers.

greater prevalence by Southern hybridisation analysis than by type-specific PCR (Tables 3.4, 3.6A and B).

**TABLE 3.9:- COMBINED HPV PREVALENCE BY SOUTHERN HYBRIDISATION ANALYSIS AND TYPE-SPECIFIC PCR ASSAYS**

Patient Group	Number (%) Lesions Positive				
	VW	K	IEC	SCC	US
<b>RAR</b>	10/14 (71)	13/30 (43)	13/31 (42)	15/29 (51)	3/21 (14)
<b>ICP</b>	6/6 (100)	2/13 (15)	5/25 (20)	4/15 (27)	1/12 (8)

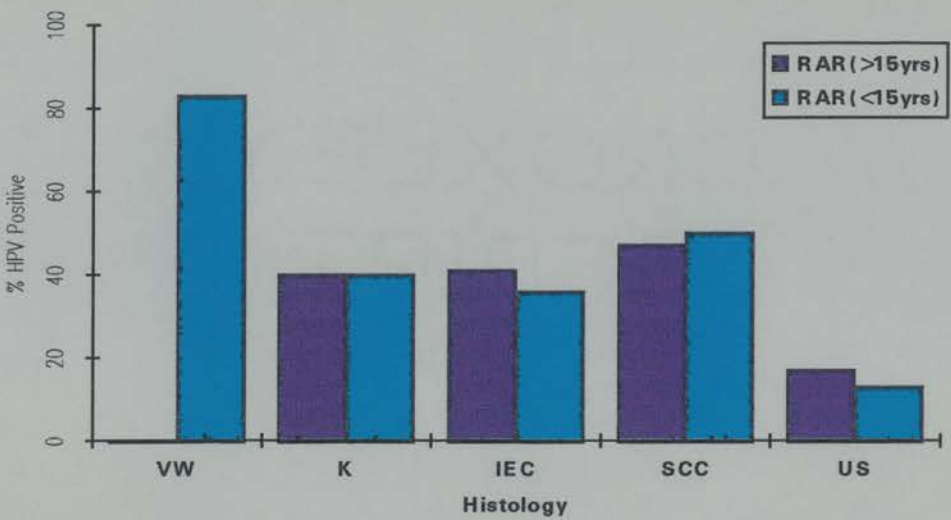
RAR = renal allograft recipient; ICP = immunocompetent patient; VW = viral wart; K = keratosis; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma; US = uninvolved, sun-exposed skin.  
 $\chi^2$  tests revealed the prevalence of HPV DNA in both SCC ( $p<0.02$ ) and IEC ( $p = 0.05$ ) was significantly greater than that in US from RARs. For both VW/RAR versus US/RAR and VW/ICP versus US/ICP,  $P<0.00003$  by  $\chi^2$  tests.

**2.7 PREVALENCE OF HPV DNA IN INDIVIDUAL PATIENTS**

Fourteen patients included in this study had graft lives of 15 years or more. A comparison of these patients with patients with a graft life of <15 years revealed no difference in the combined prevalence of HPV DNA detected by Southern hybridisation analysis and PCR (Figure 3.9). Seven RARs (patients 16, 20, 23, 34, 54, 26 and 64) were identified who appeared to have an increased susceptibility to the development of pre-malignant and malignant skin tumours (Tables 3.2A and B). The majority of these patients were found to have multiple HPV infected lesions exhibiting different HPV types (Table 3.8). Although there was no specific pattern or combination of HPV types in these patients, the prevalences of HPV DNA in keratoses (8/16 (50%)), IECs (10/22(45%)) and SCCs (14/27(52%)) from these 7 patients were increased in comparison to that observed in keratoses (4/14(29%)), IECs (2/9 (22%)) and SCCs (0/2(0%)) from 45 RARs with single, or few skin neoplasms (Figure 3.9). In

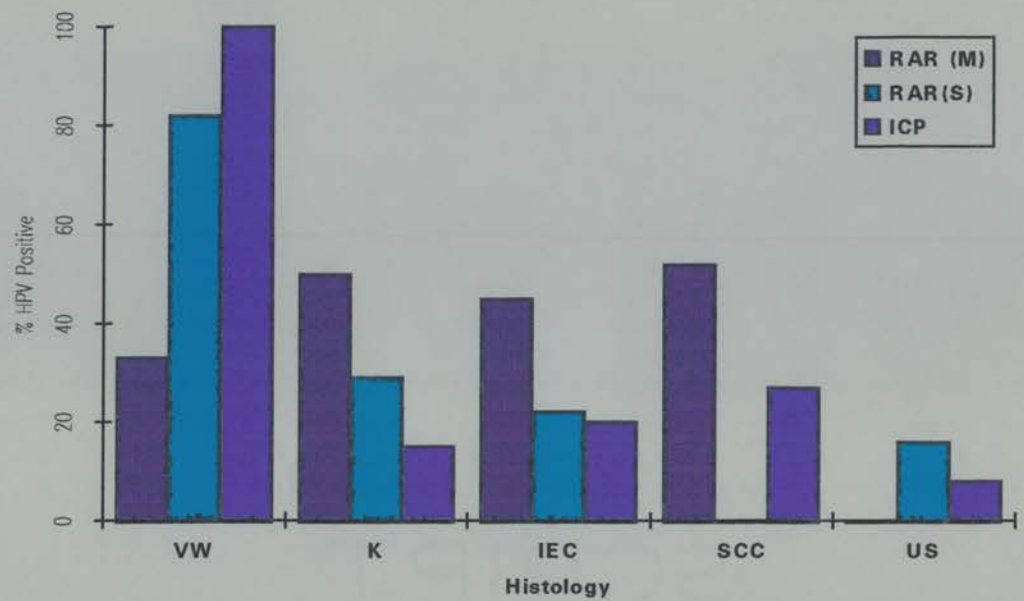
fact, the level of HPV positivity in RARs with a small number of skin neoplasms was more similar to the overall HPV prevalence in ICPs (Figure 3.10 and Table 3.9). The combined HPV positivity of keratoses+IECs+SCCs for 5 (out of 7) patients with multiple skin cancers was found to be greater than the 24% (6/25) observed for the RAR population with single or few skin tumours: 3/11(27%) for patient 16, 7/10(70%) for patient 20, 3/11(27%) for patient 23, 6/13(46%) for patient 26, 5/9(56%) for patient 34, 5/8(62%) for patient 54 and 5/10(50%) for patient 64. This difference reached statistical significance in patient numbers 20 ( $p < 0.02$  by  $\chi^2$  test) and 54 ( $p < 0.05$  by  $\chi^2$  test).

**FIGURE 3.9:- HPV DNA PREVALENCE IN PATIENTS WITH SKIN CANCER FOR  $\geq 15$  YEARS AND FOR  $<15$  YEARS**



RAR( $\geq 15$  yrs) = renal allograft recipients with renal transplants for  $\geq 15$  yrs; RAR( $< 15$  yrs) = renal allograft recipients with transplants for  $< 15$  yrs; VW = viral wart; K = keratoses; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma; US = uninvolved, sun-exposed skin.

**FIGURE 3.10 :- HPV PREVALENCE IN RARs WITH AND WITHOUT MULTIPLE SKIN LESIONS**



RAR(M) = renal allograft recipients with multiple cutaneous neoplasms;  
RAR(S) = renal allograft recipients with single or few skin neoplasms;  
ICP = immunocompetent patients; VW = viral wart; K = keratoses;  
IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma; US = uninvolved, sun-exposed skin.



### **3: P53 ACCUMULATION AND MUTATION IN CUTANEOUS NEOPLASIA FROM RENAL ALLOGRAFT RECIPIENTS**

#### **3.1 OPTIMISATION OF IMMUNOCYTOCHEMICAL TECHNIQUES**

The PAb1801 epitope on p53 is destroyed by formaldehyde fixation (Banks et al, 1986), therefore, in the majority of cases the superior antibody MAb Do-7, which recognises a formalin fixation resistant epitope of p53, was employed (Vojtesek et al, 1992). Experiments were initially carried out to optimise the immunocytochemical detection of p53 in formalin and PLPD fixed material using this new monoclonal antibody. These preliminary investigations indicated that MAb Do-7 stained p53 in sections fixed in both formalin and PLPD at an optimal dilution of 1:100. Clear immune-precipitate was present in PLPD fixed sections following a 1 hour incubation with MAb Do-7. However, overnight incubation was required for a similar clarity of staining in formalin fixed material. A comparison of the prevalence of p53 immunoreactivity in RAR lesions fixed in formalin and PLPD showed closely similar data, thus permitting the results from both fixatives to be combined (Table 3.10). A comparison of the specificity and sensitivity of MAb Do-7 and PAb1801 was also carried out on 74 PLPD fixed lesions. In the majority of cases (57), the grade of staining was identical when using the two antibodies and within each section both antibodies reacted with similarly located cells. However, a discrepancy in positivity between MAb Do-7 and PAb1801 was found in 3 cases. One US showed no p53 staining when MAb Do-7 was employed and grade 2 staining when PAb1801 was used as the primary antibody, whereas 2 cases (an AK and an SCC) were negative when treated with PAb1801 but showed grade 3 staining with MAb Do-7. There were also discrepancies in the grade of staining between MAb Do-7 and PAb1801 in that 8 cases showed a higher grade of staining when treated with MAb Do-7 as opposed to PAb 1801, while 6 cases showed more positive cells when treated with PAb1801 compared to MAb Do-7. Overall, MAb Do-7 gave a more intense precipitate than PAb1801

although some minor variation in intensity occurred between assays. The results presented are those obtained from MAb Do-7 staining.

**TABLE 3.10 : A COMPARISON OF THE PREVALENCE OF ACCUMULATED P53 IN CUTANEOUS LESIONS FROM RARs FIXED IN FORMALIN AND PLPD**

	No. Lesions (%) Demonstrating Accumulated p53#				
FIXATIVE	US	VW	K	IEC	SCC
Formalin	0/1(0)	2/14(14)	9/21(43)	10/17(59)	10/19(53)
PLPD	5/20(25)	1/7(14)	8/20(40)	12/17(70)	12/20(60)

# = Sections with staining in >10% of nuclei in the lesion (grades 2 and 3) were scored as positive. PLPD = periodate-lysine-paraformaldehyde-dichromate; US = uninvolved, sun exposed skin; VW = viral wart; K = verrucous and actinic keratoses; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma.

**3.2 P53 ACCUMULATION IN CUTANEOUS LESIONS FROM RARs AND ICPs**

A total of 156 biopsies from RARs and 80 from ICPs were screened for the presence of Accumulated p53 using MAb Do-7 (Table 3.11, Figure 3.11). In both populations, over 50% of IECs and SCCs exhibited p53 immunoreactivity in >10% of cells (grades 2 and 3). Accumulated p53 was also demonstrated in dysplastic keratoses from RARs (17/41(41%)) but the prevalence was lower than in IECs (22/34(65%)) and SCCs (22/39(56%)) and decreased with the decreasing level of dysplasia. A comparison of the results from IECs and SCCs with those from keratoses, VWs (3/21(14%)) and uninvolved skin (5/21(24%)) revealed that in RARs, significantly more IECs than keratoses (p<0.05 by  $\chi^2$  test) and significantly more IECs and SCCs than uninvolved skin (p<0.05 by  $\chi^2$  test) and VWs (p<0.01 by  $\chi^2$  test) exhibited accumulated p53. A similar trend was revealed in ICPs although a lower proportion of cases stained positive for p53. The differences between SCCs or IECs and keratoses or US were not



statistically significant in the immunocompetent population. Overall, the grade of staining within cutaneous neoplasia from RARs and ICPs correlated positively with the degree of dysplasia present (Figure 3.11). However, sparse nuclear staining, involving less than 10% of cells (grade 1), was observed in 10/39(25.6%) SCCs from RARs compared to 1/17 (6%) SCCs from ICPs and 3/34 (9%) IECs from RARs.

**TABLE 3.11 - PREVALENCE OF ACCUMULATED P53 IN CUTANEOUS LESIONS FROM RARS AND ICPs**

	No. Lesions (%) Demonstrating Accumulated p53#				
PATIENTS	US	VW	K	IEC	SCC
RAR	5/21(24)	3/21(14)	17/41(41)	22/34(65)	22/39(56)
ICP	3/12(25)	0/7(0)	4/16(25)	15/28(53)	9/17(53)

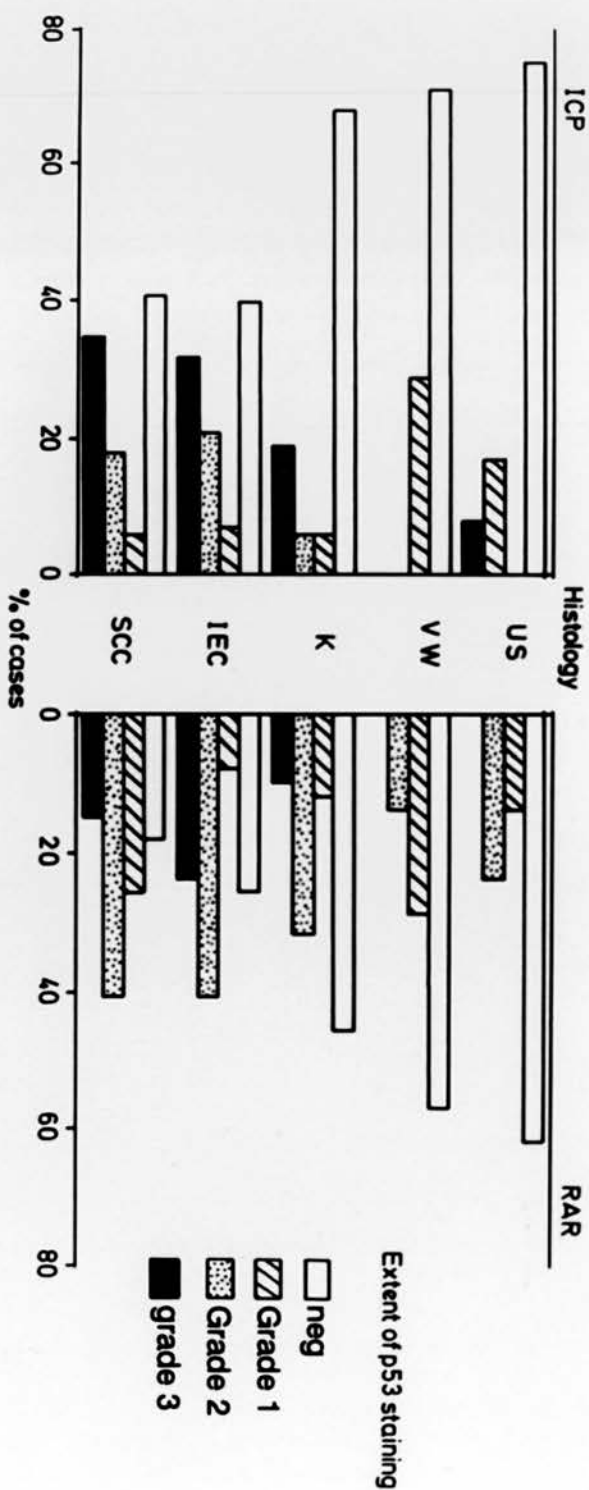
# Sections with staining in >10% of nuclei in the lesion (grades 2 and 3) were scored as positive. RAR = renal allograft recipients; ICP = immunocompetent patients; VW = viral wart; K = keratoses; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma; US = uninvolved, sun exposed skin.

$\chi^2$  tests revealed significant differences of  $p < 0.05$  for comparisons of SCC/RAR with US/RAR;  $p < 0.05$  for IEC/RAR versus US/RAR and IEC/RAR versus K/RAR;  $p < 0.01$  for comparison of IEC/RAR with US/RAR;  $p < 0.01$  for comparison of SCC/RAR with US/RAR.

**3.3 DISTRIBUTION OF ACCUMULATED P53**

In both RARs and ICPs, immunostaining of lesions was confined to nuclei of dysplastic epithelial cells and was most abundant in areas of severe dysplasia (Figure 3.12). However, some variability in staining among dysplastic cells within the same section was frequently observed. Within keratoses and IEC lesions, staining was generally strongest in basal epithelial layers, particularly at sites of basal budding where dysplastic changes were most severe (Figure 3.12A and B). This was particularly

**FIGURE 3.11:- EXTENT OF P53 STAINING IN CUTANEOUS LESIONS FROM RARS AND ICPS**



Grade 1 = <10% of cells, grade 2 = 10-50% of cells, grade 3 = >50% of cells in a lesion showing positive nuclear staining by immunocytochemistry; neg = negative by immunocytochemistry; ICP = immunocompetent patients; RAR = renal allograft recipients; US = uninvolved skin; VW = viral warts; K = keratoses; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma.

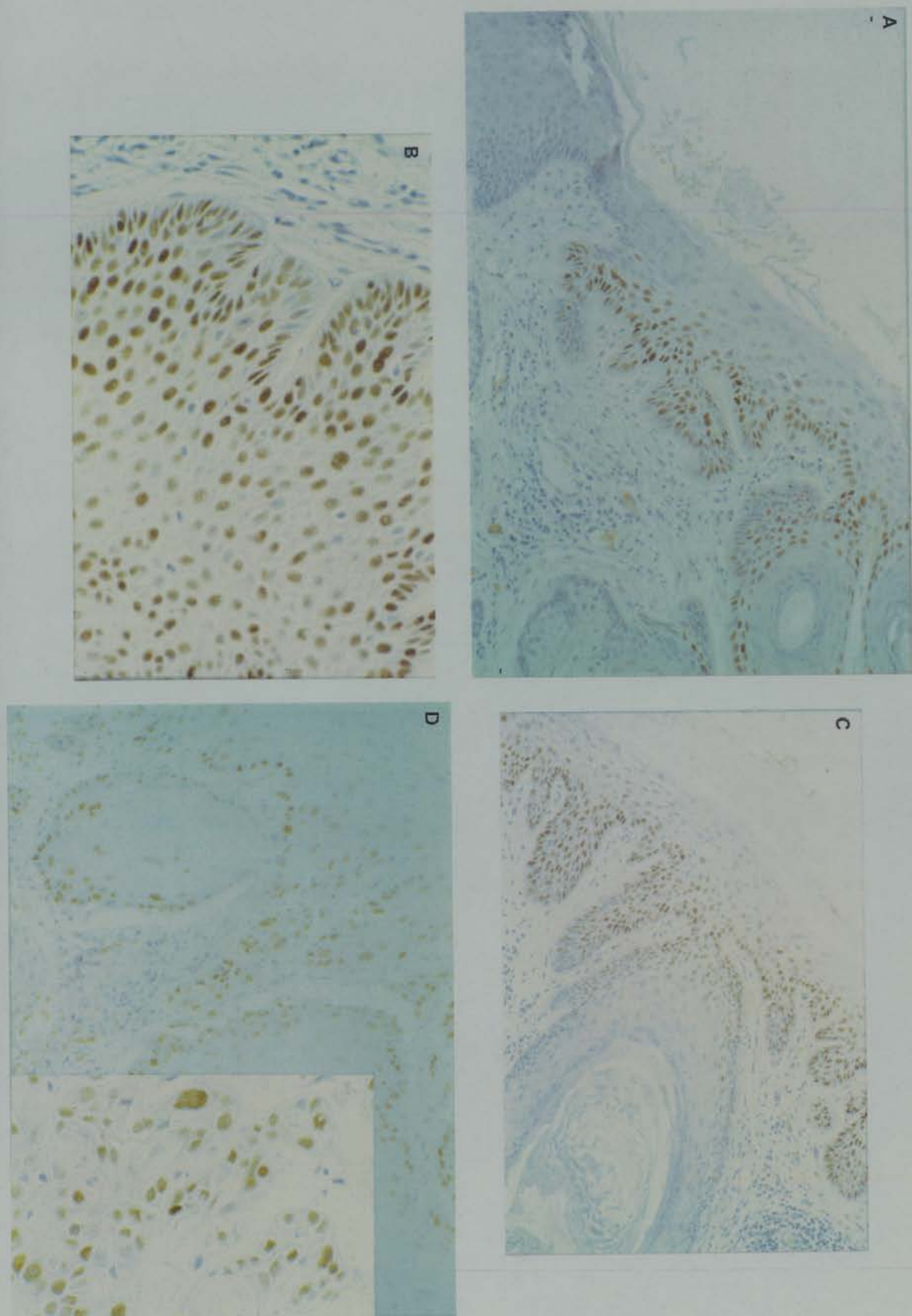
notable in keratoses exhibiting actinic features. In dysplastic keratoses and IECs, acantholysis and suprabasal clefting were also observed to correlate with strong p53 staining. Even in cases with extensive proliferation of p53 positive keratinocytes, the specialised lining cells of skin appendages, such as the hair follicle, always remained negative, with staining confined to the surrounding dysplastic cells (Figure 3.12C). Whilst the majority of SCCs showed stabilised p53 (Figure 3.12D), there was a tendency for greater positivity to occur in less well differentiated lesions. In those SCCs showing some degree of differentiation, the positive cells were located mainly in the basal layer of the keratinising tumour mass. In 5 cases of SCCs, p53 was detected in dysplastic basal cells and overlying IEC but not in contiguous tongues of invasive carcinoma. Seventeen cases of IEC/SCC contained adjacent areas of normal epidermis which always remained unstained (Figure 3.12A). The positive staining in non-lesional, sun-exposed skin was light in intensity and predominantly basal in location in cells exhibiting only mild dysplastic change.

#### **3.4 p53 IMMUNOREACTIVITY IN CUTANEOUS LESIONS FROM PATIENTS WITH DIFFERING SUSCEPTIBILITY TO NEOPLASIA**

The 66 IECs/SCCs investigated for accumulated p53 were collected from 18 RARs. A comparison of the results for RARs with multiple skin cancers (see Results Section 1.1) with those for RARs with few, or no skin cancers, revealed no difference in the prevalence of immunoreactive p53. Similarly, the percentage of immunocytochemically positive IECs and SCCs for each individual patient with multiple skin cancers was comparable to the percentage of p53 positive IECs and SCCs from RARs with few skin malignancies. There was also no difference in the number of cutaneous lesions from RARs with graft duration of  $\geq 15$  years and RARs with graft duration of  $< 15$  years exhibiting accumulated p53.

**Figure 3.12:-** Histological distribution of accumulated p53. (A) Severely dysplastic keratosis (right) is associated with strong p53 immunostaining as compared with negative normal epidermis (left). (B) p53 immunostaining is localised to the dysplastic basal cells in actinic keratosis. (C) Dysplastic basal cells are positive for p53 while the specialised cells in appendages are negative. (D) Nuclear localisation of p53 in an invasive squamous cell carcinoma from a RAR. p53 immunocytochemistry was performed using PAb Do-7 and a standard ABC /horseradish peroxidase technique.

**FIGURE 3.12: HISTOLOGICAL DISTRIBUTION OF ACCUMULATED P53**



### **3.5 p53 IMMUNOREACTIVITY AND SUN EXPOSURE**

While the majority of IECs/SCCs from both RARs and ICPs arose on sun exposed body sites, in 4 out of 44 cases showing grade 2 or 3 staining for p53, the site of the lesion suggested that UV light was not a significant aetiological factor in its development. p53 immunoreactivity was observed in a similar proportion of SCCs from patients with and without a history of high sun exposure.

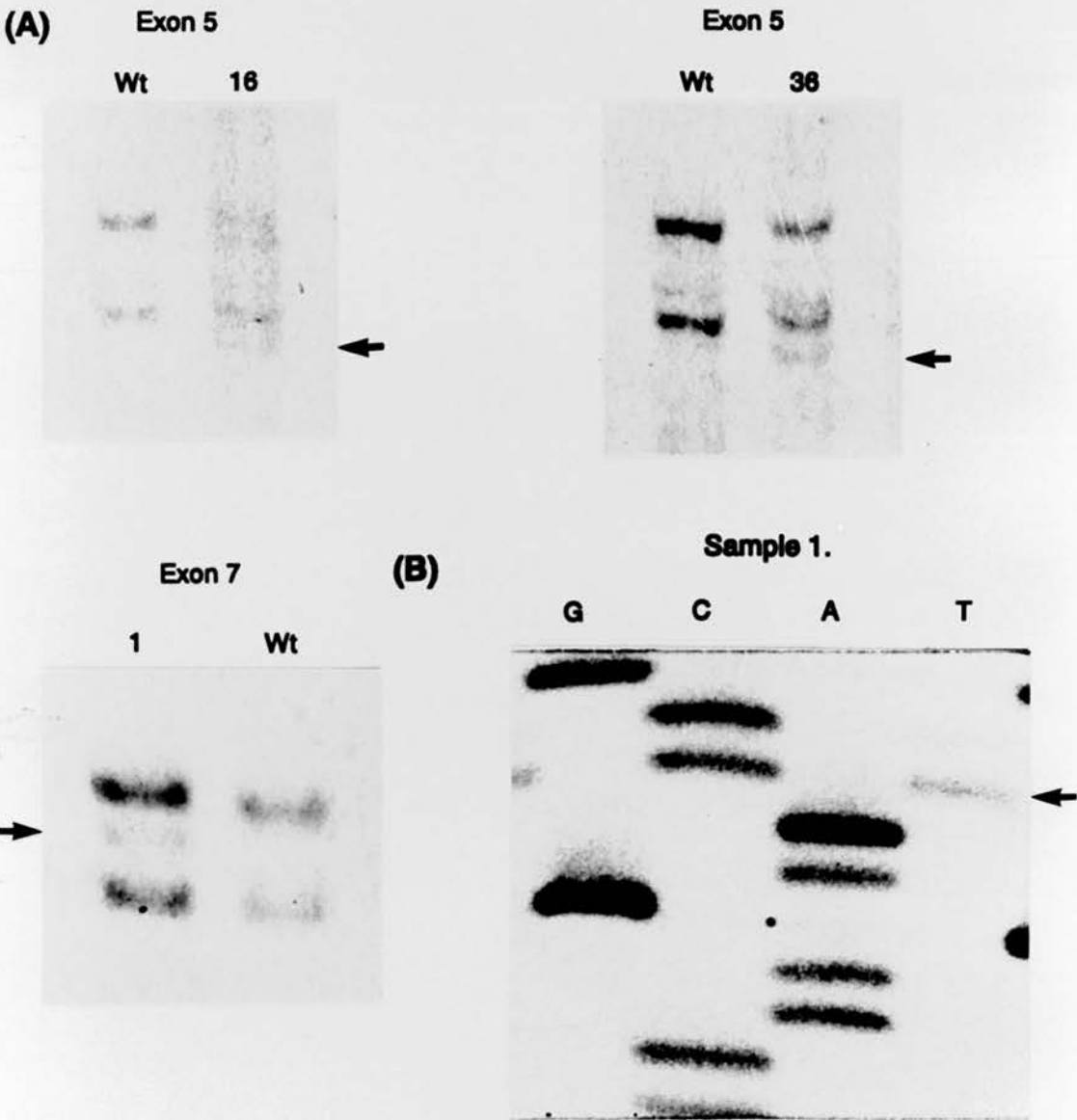
### **3.6 p53 MUTATIONS IN CUTANEOUS MALIGNANCIES**

SSCP analysis was used to investigate 28 IECs/SCCs, from 8 RARs and 12 ICPs, for the presence of p53 mutations in exons 5 to 8 of the p53 gene (Table 3.12 and Figure 3.13). Matched uninvolved skin specimens from 7 RARs and 5 ICPs were also investigated. Overall, a similar number of SCCs (3/15(20%)) and IECs (3/13(23%)) displayed p53 SSCP. Three of the SSCPs were in exon 7, two in exon 5 and one in exon 8. In contrast, no SSCPs were detected in 12 uninvolved skin samples, including 4 from patients exhibiting SSCPs in skin malignancies. A comparison of the results for RARs and ICPs revealed mutated p53 was present in more SCCs from RARs (3/9) than from ICPs (0/6). However, the number of lesions investigated was too small to determine the significance of this difference. Direct DNA sequencing of one SCC with an SSCP mutation in exon 7 revealed a C-T transition at codon 248 (Figure 3.13). 5 of the 6 IECs/SCCs exhibiting mutated p53, including the SCC with a mutation at codon 248, arose on sun-exposed body sites, implicating UV radiation as a possible causative factor. However, an IEC with an SSCP in exon 7 of the p53 gene was removed from the trunk of a patient with low sun exposure which is presumed to be a non sun-exposed site.



**Figure 3.13:-** (A) Examples of SSCP mutations in exons 5 and 7 of p53 in cutaneous malignancies from renal allograft recipients and immunocompetent patients. Using SSCP analysis, a single base change, such as a point mutation, is visualised as a band of altered migration (as indicated by arrows) in a polyacrylamide gel. Samples 1 and 16 = squamous cell carcinomas from renal allograft recipients, both with grade 3 staining by immunocytochemistry; sample 36 = an intraepidermal carcinoma from an immunocompetent patient with grade 2 staining by immunocytochemistry. (B) Direct DNA sequencing of exon 7 from sample 1 showing a C - T transition at codon 248 of p53.

**FIGURE 3.13:- P53 MUTATIONS IN CUTANEOUS MALIGNANCIES OF RENAL ALLOGRAFT RECIPIENTS**



### **3.7 SSCP ANALYSIS OF P53 IMMUNOPOSITIVE AND IMMUNONEGATIVE LESIONS**

15 of the 28 IECs/SCCs analysed by SSCP were immunopositive (grades 2 and 3) while 6 were immunonegative and 7 showed grade 1 staining (Table 3.12 and Figure 3.14). A relationship was observed between the number of malignancies displaying p53 SSCPs and the grade of p53 staining by immunocytochemistry in that 5/9 (56%) of grade 3, 1/6(17%) of grade 2 and none of grade 1 lesions showed SSCPs. No SSCPs were detected in immunonegative malignancies. With one exception, all cases with SSCP detectable p53 mutations showed grade 3 positivity by immunocytochemistry, irrespective of the exon involved.

**TABLE 3.12 - SSCP ANALYSIS OF IMMUNOPOSITIVE AND IMMUNONEGATIVE TUMOURS FROM RARS AND ICPS**

Patients	Histology	Extent Of p53 Immunoreactivity And SSCP Positivity*			
		Neg	grade 1	grade 2	grade 3
RAR	SCC	0/1	0/3	0/2	3/3
	IEC	0/3	0/1	0/1	1/2
	US	0/5	0/2	-	-
ICP	SCC	-	0/2	0/2	0/2
	IEC	0/2	0/1	1/1	1/2
	US	0/5	-	-	-

SSCP = single strand conformational polymorphism; \* = extent of p53 immunoreactivity assessed as grade 1 = < 10%, 2 = 10 - 50%, and 3 = > 50 % of cells in a lesion showing positive nuclear staining. RAR = renal allograft recipients; ICP = immunocompetent patients; SCC = squamous cell carcinoma; IEC = intraepidermal carcinoma; US = uninvolved, sun exposed forearm skin.

## **4 : P53 ABNORMALITIES AND THE PRESENCE OF HPV DNA**

### **4.1 HPV STATUS AND ACCUMULATED P53**

One hundred and twenty eight biopsies from RARs and 75 from ICPs were screened for both p53 immunoreactivity and the presence of HPV DNA (Table 3.13). Overall, no clear relationship was observed between the presence or extent of accumulated p53 and HPV DNA in pre-malignant or malignant cutaneous lesions from RARs or ICPs. However, a statistical relationship was found between the presence of HPV DNA and accumulated p53 in VWs from RAR ( $p < 0.05$  by  $\chi^2$  test) but this reflects only one p53 immunopositive VW out of 10 that were HPV positive and no p53 immunopositive VWs that were HPV negative. Therefore, this finding is probably not biologically significant. The prevalence of the specific HPV types 1,2,5,8,6,11,16 and 18 was also too low to determine whether any correlation existed between these HPV types and p53 immunoreactivity (Table 3.14).

### **4.2 HPV STATUS AND MUTATED P53**

SSCP analysis was carried out on 12 HPV positive and 16 HPV negative malignancies. These included 3 cases exhibiting HPV 2 DNA, 1 HPV 5 DNA and 1 HPV 8 DNA. p53 mutations were detected in 3 of the HPV positive and 3 of the HPV negative malignancies. Neither the HPV 5 nor the HPV 8 positive cancers showed mutated p53, however, an exon 7 mutation was found in an SCC containing HPV 2 DNA.

**TABLE 3.13 - RELATIONSHIP BETWEEN THE PRESENCE OF HPV DNA AND ACCUMULATED P53 IN CUTANEOUS LESIONS**

Histology	RAR				ICP			
	HPV+		HPV-		HPV+		HPV-	
	p53 +	p53-	p53+	p53-	p53+	p53-	p53+	p53-
US	0/3	3/3	5/16	11/16	1/1	0/1	2/11	9/11
VW	1/10 <sup>#</sup>	9/10 <sup>#</sup>	0/5	5/5	1/5	4/5	1/1	0/1
K	4/10	8/10	6/19	13/19	0/3	3/3	3/11	8/11
IEC	8/12	4/12	13/20	7/20	2/4	2/4	13/24	11/24
SCC	7/15	8/15	10/16	6/16	3/5	2/5	4/10	6/10

p53+ = Sections with staining in >10% of nuclei in the lesion (grade 2 and 3) were scored as positive. RAR = renal allograft recipient; ICP = immunocompetent patient; US = uninvolved, sun exposed skin; VW = viral wart; K = keratoses; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma.  
# = p<0.05 ( $\chi^2$  test)

**TABLE 3.14 - RELATIONSHIP BETWEEN THE PRESENCE OF SPECIFIC HPV TYPES AND ACCUMULATED P53 IN CUTANEOUS LESIONS**

HPV Type	No. HPV Positive Lesions* Exhibiting Stabilised p53				
	VW	K	IEC	SCC	US
HPV1	0/0	1/2	1/1	1/1	0/0
HPV2	0/5	0/2	2/3	2/3	1/1
HPV5	1/3	0/3	1/1	0/0	0/2
HPV8	0/0	0/0	0/0	1/1	0/0
HPV6	0/1	0/0	0/0	1/1	0/0
HPV11	1/2	0/0	0/0	0/0	0/0
HPV16	0/0	1/1	1/2	1/1	0/1
HPV18	0/0	0/0	0/0	0/0	0/0

The results are a combination of data from renal allograft recipients and immunocompetent patients. \* = As detected by type specific PCR assays. US = uninvolved, sun exposed skin; VW = viral wart; K = keratoses; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma.

## **CHAPTER 4:** **DISCUSSION**



## **CHAPTER 4: DISCUSSION**

### **1: CUTANEOUS NEOPLASIA IN RENAL ALLOGRAFT RECIPIENTS**

#### **1.1 SUSCEPTIBILITY TO CUTANEOUS NEOPLASIA IN RARs**

The majority of premalignant and malignant skin lesions collected during this study were derived from a small number of RARs, implying that some transplant patients have an increased susceptibility to the development of skin cancer. Longer graft survival times have previously been associated with a greater risk of cutaneous malignancy following renal transplantation (Benton et al, 1992). However, this study found that some patients who have had transplants for 15-21 years developed no cutaneous neoplasms over the 4 year sample collection period whereas IECs and SCCs occurred in patients with short graft lives, sometimes of only 2 years. This suggests that although graft duration may be important in skin cancer development in RARs, other factors are involved.

The role of immunosuppressive treatment in the development of cutaneous cancers in RARs has been investigated by comparing the incidence of carcinomas in patients receiving corticoids and/or azathioprine with the incidence in those receiving cyclosporin A (Shuttleworth et al, 1989; Bunney et al, 1990; Sheil et al, 1991). While some of these studies found that patients treated with cyclosporin have a reduced risk of developing cutaneous malignancies, this could not be confirmed by others. Immunosuppressive drugs *per se* are unlikely to play a role in increased cancer susceptibility in this series of patients since most of the long term transplant recipients received similar drug regimes. However, RARs with dysplastic lesions are reported to have higher levels of the active azathioprine metabolite 6-thioguanine in their erythrocytes than matched control subjects without lesions, raising the possibility that cancer susceptibility may reflect differences in levels of immunosuppression associated

with intrinsic differences in the way RARs metabolise immunosuppressive drugs (Lennard et al, 1985).

A role for sun exposure in the aetiology of cutaneous tumours in RARs was confirmed by the findings of this study that VWs and skin cancers arose predominantly on sun-exposed body sites and that the majority of RARs developing cutaneous malignancies had been exposed to moderate to high levels of UV light. However, patients were identified who developed multiple skin malignancies yet had a low sun exposure or malignancies on non-exposed sites and others were found to have had high sun exposure (who were matched for graft survival time) but no cutaneous cancers. UV radiation from sun exposure is undoubtedly playing a role as a carcinogen, but factors other than sun exposure also contribute to an increased susceptibility to the development of skin cancers in some allograft recipients. It should be noted that the assessment of sun exposure in this study was subjective and non-verifiable. Recent data suggest that sun exposure in the first 30 years of life is a critical factor determining the future risk of developing cutaneous SCCs (Bouwes Bavnick et al, 1993a). Therefore, in order to assess accurately the contribution of UV radiation to skin carcinogenesis of RARs, a more detailed survey would be required including skin type, very detailed history of sun exposure prior to transplantation and recent episodes of sun burn.

The distribution of cutaneous neoplasia and the early age of cancer development observed in this RAR population confirms previous studies of the clinical profiles and characteristics of transplant patients (Hoxtell et al, 1977; Blohme & Larko, 1984; Sheil et al, 1985; Shuttleworth et al, 1987; McLelland et al, 1988; Hartevelt et al, 1990). The histological overlap between viral and malignant features also confirms previous work on cutaneous neoplasia from RARs (Blessing et al, 1989). This combination of the importance of duration and type of immunosuppression, clinically identified acceleration of cutaneous neoplasia and histological demonstration of viral features, all

highlight the importance of investigating the viral contribution in the development of these neoplasms.

## **2: HPV AND CUTANEOUS CARCINOGENESIS OF RENAL ALLOGRAFT**

### **RECIPIENTS**

#### **2.1 PREVALENCE OF HPV DNA IN SPECTRUM OF CUTANEOUS NEOPLASIA IN RARS**

Compelling evidence exists for a contributory role for high risk genital HPV types 16 and 18 in the development of SCC of the genital tract (Arends et al, 1990, 1991 and 1993; zur Hausen, 1991; Lorincz et al, 1992). Similarly, in EV the role of HPV 5 and 8 in the aetio-pathogenesis of cutaneous SCC is suggested by their presence in over 90% of cancers (Orth et al, 1979; Orth, 1986; Fuchs & Pfister, 1990). The progression of papillomas to alimentary tract carcinomas in bracken eating cattle is strong evidence that papillomaviruses also play a role in naturally occurring animal malignancies (Jarrett et al, 1984; Campo et al, 1985; Pennie et al, 1992 and 1993). However, the stage at which papillomaviruses act in the neoplastic process appears to differ in these models of viral carcinogenesis. For example, in cervical neoplasia, the prevalence of HPV (of specific high risk types) increases throughout the cervical intraepithelial neoplasia spectrum implying that HPV plays a role in the malignant progression of cervical cancers (Stanley, 1990; Arends et al, 1991 and 1993; Lorincz et al, 1992). This is in contrast to the bovine model where there is a decrease in the number of lesions exhibiting BPV DNA as papillomas undergo malignant progression to carcinomas, in which no viral particles are detected suggesting a "hit and run" mechanism of PV involvement (Jarrett et al, 1984; Campo et al, 1985). Skin cancers from EV patients exhibit increased prevalences of specific, high risk, HPV types compared to benign and premalignant lesions from these patients, suggesting that the role of HPV in the cutaneous neoplastic process of EV is similar to that of cervical carcinogenesis (Orth et al, 1979; Pfister et al, 1981 and 1983a; Ostrow et al, 1982; Fuchs & Pfister, 1990). In order to investigate the role of HPV in cutaneous carcinogenesis of RARs, a large unselected series of cutaneous lesions from

throughout the neoplastic spectrum of RARs and ICPs were investigated for the presence of HPV DNA.

The prevalence of HPV positivity was found to be closely similar in premalignant (dysplastic) and malignant skin lesions from RARs; 43% of keratoses, 42% of IECs and 51% of SCCs. This level of HPV positivity was significantly greater than the 14% observed in non-involved, sun-exposed skin from RARs and substantially increased in comparison to that of cutaneous neoplasia from ICPs; 15% of keratoses, 20% of IECs and 27% of SCCs. These data provide strong evidence that HPVs play an early but persistent role in cutaneous carcinogenesis of RARs. This pattern of HPV positivity differs from the above (bovine alimentary and cervical/EV) models of PV induced carcinogenesis implying that the putative role is neither as an initiating agent nor as an inducer of malignant progression (see Section 4 for further discussion).

The prevalence of HPV DNA in carcinomas reported here is in broad agreement with that of Soler et al, (1992) and Euvrard et al, (1993) who detected HPV DNA sequences in 7 of 19 and 14 of 30 SCCs from RARs respectively. In contrast, Barr et al, (1989) found HPV DNA in 60% of SCCs from RARs and Dyll-Smith et al, (1991) detected no HPV DNA in 235 SCCs from RARs. However, it is inappropriate to compare the results of this investigation with those of the two above mentioned studies due to differences in the sensitivity and specificity of the detection methods employed and differences in sample size studied.

## **2.2 SPECIFIC HPV TYPES IN CUTANEOUS NEOPLASMS FROM RARS**

In the bovine, cervical and EV models of papillomavirus induced carcinogenesis, only some of the infecting virus types appear to have the potential for oncogenicity. This is exemplified by skin cancers developing in cutaneous neoplasms of EV patients. While the skin of affected individuals harbours a multiplicity of HPV types, SCCs arise

mostly in lesions containing HPV types 5 and 8 (Orth et al, 1979; Lutzner et al, 1980; Ostrow et al, 1982; Pfister et al, 1983a; Orth, 1986). Similarly, there is a predominance of HPV 16 and 18 and a near absence of HPV 6 and 11 in invasive cervical cancers (Arends et al, 1990 and 1991; Lorincz et al, 1992) and of the BPV types that infect cattle, only BPV 4 is associated with malignant progression (Lynch, 1982; Quan & Moy, 1991).

In this study, restriction pattern analysis of samples found to contain HPV DNA by low stringency Southern hybridisation indicated that multiple different HPV types are present in both premalignant and malignant skin tumours from RARs. Some of these appeared to be related to common cutaneous HPV types while others gave *Pst*I restriction patterns comparable to those of EV associated HPV types. Similarly, "high risk" (HPV 5, and 16) and "low risk" (HPV 1, 2 and 6) cutaneous and mucocutaneous HPV types were detected in a small number of IECs/SCCs from RARs by type specific PCR. These data suggest that numerous "high risk" and "low risk" HPV types may contribute to the development of skin cancers in RARs which is unlike the other models of PV induced carcinogenesis. These results are in keeping with those of Soler et al, (1992), Euvrard et al, (1993) and Tieben et al, (1994) who also detected different EV-related HPV types in skin carcinomas from transplant patients.

Type specific PCR, using highly sensitive primers, indicated that only a small proportion of skin lesions from both RARs and ICPs exhibit HPV 5 or 8 DNA. This is in contrast to previous findings from South East Scotland (Barr et al, 1989) where 15 out of 25 SCCs were found to contain HPV 5/8 DNA. The study of Barr et al used mostly dot blotting techniques to detect HPV 5/8 DNA which does not exclude the possibility of cross hybridisation with other EV-associated HPV types. Other recent studies have also been unable to detect HPV 5 or 8 DNA in significant numbers of skin malignancies from transplant patients (Soler et al, 1992; Euvrard et al, 1993; Tieben et



al, 1994). Therefore, the balance of evidence now suggests that HPV 5 and 8 are present relatively infrequently in tumours from RARs (Lutzner et al, 1983; Rudlinger et al, 1986; Rudlinger & Grob, 1989; Van der Leest, 1987).

One of the important features of papillomaviruses is their propensity to infect squamous epithelium at specific sites. Although the mucocutaneous HPV type 16 DNA has previously been detected in cutaneous neoplasia from RARs and ICPs, most studies suggest that such findings are confined to SCCs of the periungual site (Stone et al, 1987; Ostrow et al, 1987 and 1989; Rudlinger et al, 1989; Eliezri et al, 1990; Ashinoff et al, 1991). In contrast, this study demonstrated anogenital HPV types in cutaneous lesions from throughout the neoplastic spectrum of RARs and at sites outwith the periungual area. Anogenital HPV types could not be detected in skin lesions from ICPs. These findings suggest that the site specific distribution of HPV is no longer observed in cutaneous lesions of RARs. Two positive SCCs, one containing HPV 6 and the other HPV 16 DNA, came from a female RAR (patient 20) who, in addition to multiple cutaneous SCCs developed SCCs of the cervix, vulva and anal canal. HPV 16 DNA has also been detected in her genital tract tumours (Alloub et al, 1989; Rudlinger et al, 1986) indicating that this patient may have a high background level of HPV infection (see Section 4.6 of the Discussion).

### **2.3 PHYSICAL STATUS OF HPV DNA IN CUTANEOUS LESIONS FROM RARS**

HPV 16 and 18 DNA are maintained mainly as extrachromosomal episomes in condyloma and mildly dysplastic genital tract lesions but are integrated into the host genome in the majority of cervical cancers. This suggests that viral integration is an important step in the malignant progression of cervical neoplasia (Fukushima et al, 1990; Cullen et al, 1991). In contrast, high copy numbers of episomal HPV 5 and 8 DNA are usually present in skin cancers of EV patients. (Yabe et al, 1989; Fuchs & Pfister, 1990). In this study, 2 VWs, a dysplastic keratosis and an SCC gave high

(>12Kb) and low molecular weight restriction fragments when digested with the single cut (or in some cases double cut) enzyme *BamHI* and the multicut enzyme *PstI*. Furthermore, amplification of the dysplastic keratosis with L1 consensus primers resulted in two specific products, one of the correct molecular weight (450bp) and another of increased molecular weight (650bp). These data raise the possibility that both episomal and integrated forms of HPV may be present in cutaneous neoplasms of RARs. However, the numbers of affected lesions were too small to determine whether integration plays a significant role. Although integration of cutaneous HPV types has rarely been observed *in vivo*, the common cutaneous HPV type 1 has previously been found integrated into the host genome at 2q33 in an experimentally generated SV40/HPV1 transformed human keratinocyte cell line (Parton et al, 1990).

Evidence from this study also suggests that the physical state of episomal HPV DNA is altered in some cutaneous lesions from RARs. First, aberrant restriction digest patterns (giving restriction fragments with a combined molecular weight of greater than 8Kb) were observed when some samples (mainly IECs/SCCs) were digested with one of *BamHI/PstI* but not the other. This may indicate the presence of mutant viral species that have lost or gained *BamHI/PstI* restriction sites or new, previously unidentified HPV types. Second, digestion of one IEC with both *BamHI* and *PstI* resulted in a single fragment of 3-3.5Kb suggesting that in this case, the major viral species was a deletant mutant of HPV. Finally, amplification of two more IECs with HPV 16 primers resulted in two specific products with molecular weights greater than the expected size. One explanation for this result is that duplication has occurred within the E6 ORF of HPV 16 in these malignancies. Consequently, it appears that RARs are not only susceptible to multiple HPV infections with rarer HPV types but that there is also greater variation within these types. The results presented here are in keeping with those of Wilson et al (1989) who demonstrated novel variants of HPV 2 in VWs from RARs. In EV, the bulk of unit length HPV molecules is typically accompanied by a

variable proportion of genomes showing various deletions or duplications (Yabe et al, 1989).

#### **2.4 OTHER HPV TYPES IN CUTANEOUS NEOPLASMS OF RARS**

Some cases found to contain HPV sequences by PCR could not be confirmed by Southern hybridisation analysis indicating that in many cases copy numbers of HPV genomes were too low to be detected by Southern analysis. Not surprisingly, the absolutely type specific assays for HPV types 1, 2, 5, 8, 6, 11, 16 and 18 did not detect other HPV types found by Southern hybridisation. Therefore, through a combination of a wide variety of HPV types involved and low copy number of HPV genomes, it is possible that this and other studies have underestimated the true prevalence of HPV in cutaneous neoplasms of RARs. In order to address this possibility, a consensus primer PCR assay was investigated. Since very little sequence information was available for skin associated HPV types, the consensus primers used in this assay were designed from genital HPV DNA sequences and had previously been shown to amplify a large range of genital associated HPV types (Manos et al, 1989). It was found that these primers detect common cutaneous and EV associated HPV types poorly, even when using cloned HPV DNA as template. Furthermore, the assay could only detect HPV DNA in a small number of samples shown to contain HPV by low stringency Southern hybridisation analysis. However, consensus primer PCR did identify HPV DNA in 2 (a VW and an SCC) of the 25 HPV "negative" skin lesions investigated, confirming the suggestion that the overall HPV prevalence given in this study is an underestimate of the true figure. Overall, it was concluded that in order to obtain meaningful results from a consensus primer PCR assay, primers must be used that are designed to detect skin associated HPV types. Additional sequence information for cutaneous HPVs has recently become available and consequently, consensus primers have been published that are reported to efficiently detect cutaneous HPV types (Snijders et al, 1991; Tieben et al, 1994).

Restriction fragment length polymorphism (RFLP) analysis of consensus primer PCR products was also investigated as a method of characterising the HPV types found by a Southern hybridisation analysis screen (Lungu et al, 1992). The RFLPs obtained from the small number of amplifiable samples were dissimilar to those of the genital HPV types published by Lungu et al, (1992) and to those of the HPV types investigated in this study. These results confirm the presence of multiple HPV types in cutaneous neoplasia from RARs. The enzyme cocktails employed by Lungu et al (1992) were designed primarily to identify genital HPV types amplified using the Manos consensus primers. These enzyme cocktails were found to be inefficient at distinguishing between cloned EV-associated HPV types and, the RFLPs obtained from other cloned cutaneous HPV types were difficult to reproduce. Once again, additional sequence information is required to design an enzyme cocktail that will efficiently distinguish between cutaneous HPV types amplified using consensus PCR primers.

**In conclusion, the similar (40-50%) prevalence of HPV DNA found in keratoses, IECs and SCCs from RARs implies that HPV acts at an early stage in cutaneous carcinogenesis of transplant patients. Multiple common cutaneous, mucocutaneous and EV-associated HPV types were detected in tumours from throughout the neoplastic spectrum of RARs, although HPV 5 and 8 were present only at a low frequency in skin cancers from both RARs and ICPs. "Low risk" and "high risk" HPV types were present in broadly equivalent numbers with no single HPV type predominating in any group of neoplasms.**

### **3: P53 IN CUTANEOUS CARCINOGENESIS OF RENAL ALLOGRAFT**

#### **RECIPIENTS**

##### **3.1 P53 ACCUMULATION AND PROGRESSION IN CUTANEOUS CARCINOGENESIS**

The exact stage at which p53 acts in the carcinogenic process appears to be characteristic for different cancers types. For example, aflatoxin in liver cancer may induce direct modifications to the p53 gene, implying that p53 mutations are an initiating event in this cancer type (Vogelstein and Kinzler, 1992b). In contrast, p53 mutations are associated with malignant conversion (from benign adenoma to invasive carcinoma) in colorectal carcinogenesis (Purdie et al, 1991; Carder et al, 1993). In HPV infected cervical cancers, mutation of the p53 gene is reported to associate with the late metastatic stage of the disease (Mitranirosenbaum & Tsvieli, 1994). Previous investigations into the prevalence of stabilised and mutated p53 in dysplastic and pre-malignant cutaneous lesions from ICPs have been inconsistent, therefore, the stage at which p53 acts in human skin carcinogenesis is presently unknown.

This study was the first to examine the role of p53 in cutaneous carcinogenesis of RARs and the first to investigate a large series of cutaneous tumours from throughout the neoplastic spectrum in ICPs. The prevalence and extent of p53 immunoreactivity increased as lesions progressed through the spectrum of neoplasia to the pre-invasive state; 14% of VWs; 24% of sun exposed skin; 41% of keratoses and 65% of IECs exhibited accumulation of p53. Topographically, there was a close correlation between the extent of staining in these lesions and the severity of dysplasia. However, a similar prevalence of immunoreactive p53 was observed in IECs and SCCs (56%) with a similar extent of staining. These results suggest that accumulation of p53 in skin carcinogenesis of both RARs and ICPs represents an important step in neoplastic progression but is not the rate limiting step in the progression from *in situ* to invasive



SCC. Other genetic events would appear to occur prior to the development of malignancy. This hypothesis is supported by the finding of Dotto et al (1988) that mutated p53, introduced into murine papilloma cell lines, accentuates the dysplastic changes in the resultant tumours *in vivo* but does not convert these tumours to malignancy. In contrast, Kemp et al (1993) demonstrated that inactivation of p53 specifically associates with the conversion of benign papillomas to SCCs in p53 deficient mice and that papillomas and carcinomas from these mice are less well differentiated than their counterparts with wild type p53. In this study, a comparison of immunopositive and immunonegative (including those with grade 1 staining) SCCs revealed no differences in the differentiation state of the lesion. This supports the hypothesis that genetic events other than accumulation of p53 are associated with loss of differentiation and development of malignancy in human SCCs.

The finding of accumulated p53 in approximately 50% of IECs and SCCs is in broad agreement with some previously reported studies where the prevalence has ranged from 15-56% for SCCs and 17-80% for IECs (Gusterson et al, 1991; McGregor et al, 1992; Ro et al, 1992; Helander et al, 1993). The trend of p53 positivity in neoplasia from throughout the histological spectrum observed in this study is similar to that demonstrated by Gusterson et al (1991) who detected p53 immunoreactivity in 0/12(0%) solar keratoses, 2/12(17%) Bowens disease (IECs) and 3/20(15%) SCCs from ICPs. By comparison, McGregor et al (1992) found a similar prevalence of stabilised p53 in solar keratoses, IECs and SCCs from ICPs and, Ro et al (1992) detected immunoreactive p53 in an increasing number of such lesions. These discrepancies may be explained by the small number of lesions investigated in one or more of the histological categories of keratoses/IEC/SCC in these previous investigations and the criteria employed for determining a positive or negative result.



### **3.2 p53 ACCUMULATION IN CUTANEOUS NEOPLASIA FROM RARS AND ICPs**

The overall prevalence and extent of stabilised p53 was higher (but not statistically significantly greater) in VWs and keratotic skin lesions of RARs compared to those of ICPs. However, a similar proportion of uninvolved skin from RARs and ICPs exhibited immunoreactive p53. One explanation for these results is that epidermal keratinocytes with proliferative activity from RARs (such as those of VWs and keratoses) have increased levels of genetic instability compared to those from ICPs and consequently acquire accumulated p53 earlier in the neoplastic spectrum. The accumulation of p53 (and associated loss of function) may in turn lead to increased dysplasia and accelerated rate of progression of these lesions (see section 4 for further discussion). This hypothesis is supported by the clinico-pathological observations that, unlike VWs from ICPs which are benign and show no evidence of dysplasia, VWs from RARs may be dysplastic and if left untreated can even progress to malignancy (Blessing et al, 1989; Benton et al, 1992). Similarly, keratoses from ICPs usually show low levels of dysplasia in the basal layer of the epithelium and rarely progress to malignancy (Marks et al, 1986 and 1989), whereas comparable lesions from RARs are usually severely dysplastic and may even show areas of *in situ* carcinoma (IEC) (Blessing et al, 1989). An alternative hypothesis is that RARs lack immune-surveillance of cells expressing abnormal p53 which results in increased incidence and clinical rate of progression of skin neoplasia. Although anti-p53 antibodies have been detected in the serum of patients with certain types of cancer (Caron De Fromental et al, 1987; Schlichtholz et al, 1992), this hypothesis is unlikely since RARs do not have an increase in common solid malignancies, such as those of the lung, breast and colon, which are also associated with accumulation of p53 (Shuttleworth et al, 1987; Alloub et al, 1989; Barr et al, 1989).

Sparse nuclear staining (involving less than 10% of cells), although observed in all histological categories, was surprisingly prevalent in SCCs from RARs. It has previously been demonstrated that epidermal keratinocytes express increased levels of wild type p53 in response to UV induced DNA damage (Hall et al, 1993). It may be possible that SCCs from RARs have a high degree of genetic damage or instability so increased numbers of cells express wild type p53 in order to undergo DNA repair (see Section 4 for further discussion). Sparse nuclear staining was only observed in a small proportion of SCCs from ICPs raising the possibility that SCCs from RARs have increased levels of genetic instability compared to those from ICP. A small number of SCCs from RARs, but none from ICPs, were found to have p53 staining in superficial dysplastic epidermis and adjacent areas of IEC but not in contiguous tongues of invasive SCC. One possible explanation for this may be that gross chromosomal deletions, involving 17p, have occurred in more invasive malignant elements, abolishing all p53 gene expression. This also raises the possibility that SCCs from RARs are more genetically unstable than SCCs from ICPs and suggests that malignant conversion of cutaneous IEC to SCC may be associated with complete deletion of p53 and the development of aneuploidy. Further studies such as static cytometric ploidy analysis, microsatellite instability or PCR allelotyping would be required to investigate possible differences in ploidy and genetic instability in these lesions. Chromosomal deletions of p53 have previously been reported in some sarcomas although overall, this is a rare phenomenon (Baas et al, 1994).

### **3.3 p53 MUTATION AND CUTANEOUS CARCINOGENESIS**

SSCP analysis demonstrated mutated p53 in approximately 20% of both IECs and SCCs, and this is substantially lower than that reported for common solid malignancies such as the colon and lung (Baker et al, 1990a; Hollstein et al, 1991). There are a number of possible explanations for this. First, the overall proportion of cases with mutated p53 may have been underestimated because of the selection of p53

immunopositive and immunonegative lesions for analysis by SSCP. However, the results presented here are in agreement with the majority of reports on p53 mutation in skin malignancies which suggests that this is not the case (Pierceall et al, 1991a; Moles et al, 1993; Burns et al, 1993; Campbell et al, 1993a). Mutations may have occurred outwith exons 5 to 8, but studies of other common cancers, including skin, suggest that this is likely to account for only a small proportion (less than 10% in cancers of the colon and lung) of cancers (Baker et al, 1990a; Brash et al, 1991; Campbell et al, 1993a). Alternatively, p53 may be inactivated by mechanisms other than mutation in cutaneous carcinogenesis, such as the binding of viral or cellular proteins (see Section 4 for further discussion). With one exception, gene mutations occurred in exons 5 and 7, consistent with the suggestion that these exons contain mutational hotspots for p53 in various forms of human malignancy (Brash et al, 1991; Hollstein et al, 1991; Pierceall et al, 1991a; Campbell et al, 1993a; Levine, 1993).

In this study, SSCP analysis was used to detect mutations in the p53 gene. This technique is based on the principle that single stranded DNA molecules take on specific secondary structures under non-denaturing conditions (Orita et al, 1989; Mashiyama, 1990; Hayashi, 1992). Molecules differing by as little as a single base substitution may form different secondary structures and therefore, migrate aberrantly in a non-denaturing, polyacrylamide gel. The sensitivity of SSCP depends on how the mutation affects the secondary structure of single stranded DNA and therefore, its electrophoretic mobility (Smith et al, 1992; Sheffield et al, 1993). Factors that are thought to influence this sensitivity include the length of the PCR fragment (150bp is reported to be the optimal length), the position of the base substitution within the fragment, the nature of the base substitution (transition versus transversion) and the sequence composition of the DNA fragment. Therefore, the precise sensitivity of this technique depends on the gene being analysed, although overall, it is reported to detect >90% of all single base substitutions in a 200 nucleotide fragment (Hayashi et al,

1992). A recent study using the same PCR/SSCP primers as this study but on colorectal cancers, where both SSCP analysis and direct sequencing were employed to detect mutated p53, indicated that 80% of p53 gene mutations can be detected by this method (Cripps et al, 1994). Another study investigating the sensitivity of SSCP with respect to contaminating normal tissue demonstrated that p53 mutations can be detected even when they comprise only 5-10% of alleles (Wu et al, 1993).

### **3.4 p53 ACCUMULATION AND GENE MUTATION IN CUTANEOUS CARCINOGENESIS**

High levels of p53 protein are a common feature of many human neoplasms and it has often been assumed that accumulated p53, visualised by immunocytochemistry, is equivalent to mutated p53. Indeed, in the majority of tumours a good correlation has been observed between the presence of immunocytochemically detectable p53 and gene mutations as determined by direct sequencing and other methods (Iggo et al, 1990; Gannon et al, 1990; Varley et al, 1991; Burns et al, 1993; Baas et al, 1994). However, this relationship is not absolute (Bodner et al, 1992; Wynford-Thomas, 1992). For example, false negatives may occur if both p53 genes have been deleted, if the concentration of stabilised protein fails to rise to detectable levels or if the configuration of the mutant protein is not recognised by the antibody. Alternatively, false positives could occur if p53 were stabilised by other mechanisms such as a defect in the degradative pathway or the binding of cellular or viral proteins.

In this study, SSCP analysis of exons 5 to 8 of the p53 gene detected mutations in 6/15 (40%) immunopositive (grade 2 and 3 staining by immunocytochemistry) malignancies, indicating that factors other than gene mutation may contribute to the accumulation of p53 during the development of at least some skin cancers. Recently, the product of the mdm-2 gene, which is overexpressed in osteosarcomas, has been shown to bind to and inactivate p53 (Momand et al, 1992; Oliner et al, 1992 and 1993). It is possible that

similar proteins are present in transformed epidermal cells that bind to and inactivate wild type p53, rendering it detectable by immunocytochemical techniques. In this context it is of interest that a cancer family syndrome has been identified where normal epidermal and mesenchymal cells express high levels of wild type p53 (Barnes et al, 1992). It may be that the germline mutation predisposing these cancer family patients to early onset of malignancies and increased levels of wild type p53 also occurs sporadically in skin cancers.

The results reported here for skin cancer are analogous to those for breast cancer where a group of tumours have been identified that show high levels of p53 protein in the absence of p53 gene mutations (Bartek et al, 1990a and b; Dunn et al, 1993). In an elegant series of *in vitro* experiments, Vojtesek & Lane (1993) demonstrated that the cell environment is critical in the regulation of p53 in breast cancer cell lines and not p53 gene mutation *per se*. An enzyme linked immunoassay has recently shown that p53 stabilisation also occurs in some colorectal adenomas in the absence of p53 gene mutation and there is evidence to suggest that p53 accumulation can arise due to the activation of other genes already implicated in cancer such as *c-myc* and *ras* (Lu et al, 1992).

In this study, several distinct patterns of p53 staining were observed in cutaneous neoplasia, a phenomenon which has been described previously (Purdie et al, 1991; Bartek et al, 1990b; Midgley et al, 1991). In some IECs/SCCs, intense immune precipitate was present in the majority of cells (grade 3) while others showed either patchy, widespread staining (grade 2) or staining in occasional cells (grade 1). This study found a direct correlation between the grade of p53 immunopositivity and the percentage of cases exhibiting mutated p53. Therefore one hypothesis, previously proposed by Hall & Lane (1994), is that these different immunochemical phenotypes represent different biological mechanisms for the accumulation of p53. In those cases



showing grade 1 staining, wild type p53 accumulation may have occurred as a natural response to genotoxic damage. Grade 2 staining may represent stabilisation of p53 by mechanisms other than mutation such as complex formation with viral or cellular proteins or reduced p53 degradation, whereas p53 mutation may result in grade 3 staining. This hypothesis is supported by the observation that breast cancer cell lines expressing high levels of wild type p53 usually show cellular heterogeneity in their pattern of p53 expression (Bartek et al, 1990a; Vojtesek & Lane, 1993). Cells transformed by *ras* and *myc* which exhibit increased expression of wild type p53, also have a heterogeneous p53 staining pattern (Lu et al, 1992). Barnes et al (1993) have recently demonstrated that the extent of p53 immunostaining is of prognostic significance in breast carcinomas. Thus, immunocytochemistry may prove to be a good screening method for assessing the functional status of p53 within dysplastic and normal cells, however, further work is required to confirm the biological basis for these different staining patterns.

**In conclusion, this study demonstrated a strong association between p53 accumulation, dysplasia and neoplastic progression in cutaneous carcinogenesis of RARs and ICPs. The prevalence and extent of p53 immunostaining was greater in pre-malignant tumours from RARs than from ICPs which may reflect the faster clinical rate of neoplastic progression towards invasive carcinoma of these lesions in allograft recipients. SSCP analysis of exons 5 to 8 of the p53 gene in cutaneous IECs and SCCs detected p53 mutations in 21% of cases. p53 gene mutations were present in only 40% of immunopositive IEC/SCC tested indicating that factors other than p53 mutation may be responsible for the accumulation of p53 in some cutaneous neoplasms. It is possible that the pattern of p53 immunostaining may reflect the biological potential of lesions.**



## **4: MODEL OF CUTANEOUS CARCINOGENESIS IN RENAL ALLOGRAFT RECIPIENTS**

Cancers do not arise *de novo* in their final form but begin as small indolent growths that gradually acquire characteristics associated with malignancy (Sidransky et al, 1992a and b). In the skin, low grade dysplasias, such as actinic keratoses, may evolve into more dysplastic lesions (*in situ* or intraepidermal carcinoma) which may eventually become invasive (SCC) (Marks et al, 1986; Milburn et al, 1988; Johnson et al, 1992; Ledo, 1992). It has been postulated that the neoplastic progression of low to high grade tumours is driven by a series of genetic alterations, with each alteration conferring a selective growth advantage over other cells (Nowell, 1976). This hypothesis is most strongly supported by mouse skin and human colon models of tumour progression where a number of the genetic changes occurring at different stages have been characterised (Vogelstein et al, 1988; Burns et al, 1991). In this study, the availability of cutaneous lesions from throughout the neoplastic spectrum has enabled investigation into the functional roles of HPV and p53 at the different stages of cutaneous carcinogenesis in RARs.

### **4.1 THE ROLE OF HPV IN CUTANEOUS CARCINOGENESIS OF RARS**

The overall finding in this study was of similar HPV prevalence throughout the spectrum of cutaneous neoplasia in RARs. Studies of the prevalence of accurately typed, specific HPV indicated that multiple different EV-associated, common cutaneous and mucocutaneous HPV types are present in cutaneous lesions from RARs with no one specific type predominating. One hypothesis to explain these results is that HPV act as tumour promoters in cutaneous carcinogenesis of RARs. In the mouse model of skin carcinogenesis, the phorbol ester class of tumour promoters act by selectively stimulating the proliferation of initiated keratinocytes while inducing differentiation of the others (Hartley et al, 1985 and 1987). Consequently, the genetic

changes generated by initiating agents, such as chemical carcinogens or UV light, are fixed irreversibly and expansion of the initiated cell population occurs. However, multiple applications of promoting agent are required over a prolonged period before papillomas develop and malignant conversion takes place. The ability to induce keratinocyte proliferation is common to all HPV types, therefore, HPV may provide a stimulus analogous to phorbol esters in cutaneous carcinogenesis of RARs (Figure 4.1). The role of promoter for HPV has previously been suggested by zur Hausen (1982). In those keratinocytes with no DNA damage, HPV infection may lead to cell proliferation, differentiation and the development of viral warts. However, the presence of long-term immunosuppressive therapy is likely to permit viral persistence (and therefore cell proliferation) over a prolonged period which, along with the DNA damaging effects of UV radiation (see Section 4.4 for further discussion), may give rise to an environment conducive to tumour induction. This hypothesis is supported by the clinico-pathological observations that RARs frequently exhibit extensive warts and verrucous keratoses as well as malignant tumours: these lesions form a seamless spectrum of histological change, with many keratoses (actinic and verrucous) showing dysplasia, and both IECs and SCCs retaining viral features. Furthermore, these immunosuppressed patients appear to have relatively high background levels of HPV indicated by the finding that 3 out of 21 (14%) biopsies from the apparently uninvolved skin of RARs contained HPV DNA. HPV encourage squamous rather than basal cell proliferation and differentiation, therefore, the finding that the usual 1:5 ratio of SCC:BCC is reversed in RARs to 15:1 further supports a role for HPV as a tumour promoter.

In the mouse model of skin carcinogenesis, specific genetic events are associated with the progression of papillomas to malignant SCCs. It is likely that in RARs, the progression from dysplastic keratoses to IECs and then SCCs involves further genetic damage and mutation/deletion of specific oncogenes or tumour suppressor genes. HPV

induced cell proliferation may increase the chance of these specific genetic events occurring. Therefore, as well as tumour induction, HPV infection may also indirectly contribute to neoplastic progression in RARs.

#### **4.2 THE ROLE OF P53 IN CUTANEOUS CARCINOGENESIS OF RARS**

The results of this study show that the largest increment in the prevalence of p53 accumulation was from keratoses to IECs in both RARs (41% for keratoses compared to 65% for IECs) and ICPs (25% in keratoses compared to 53% in IECs). Furthermore, in RARs this difference was statistically significant ( $p < 0.05$  by  $\chi^2$  test). These data suggest that inactivation of p53 acts at the point of progression from dysplastic keratosis to IEC in cutaneous carcinogenesis of both RARs and ICPs (Figure 4.1). The implication of this data is that IECs evolve through clonal expansion of cells that have previously acquired abnormal expression of p53 and therefore a selective growth advantage. Sidransky et al (1992b) have also identified p53 as a selectable gene and demonstrated that clonal expansion of p53 mutated cells is associated with progression of low grade to high grade astrocytomas.

Within some immunopositive IECs and SCCs there was a marked cell to cell heterogeneity in p53 immunostaining which may contradict the concept of clonal origin and expansion. Evidence from this study suggests that a factors other than p53 gene mutation are responsible for the accumulation of p53 in the majority of IECs/SCCs exhibiting this heterogeneous staining pattern (see section 3.4 of the Discussion). One possible explanation for the heterogeneity is that abnormal p53 accumulation, like wild type p53, is regulated during the cell cycle and consequently only cycling tumour cells express increased levels of p53 (Reich and Levine, 1984; Shaulsky et al, 1990; Bischoff et al, 1990a; Steinmeyer et al, 1990; Perry & Levine, 1993). This hypothesis is supported by the finding that p53 immunostaining was confined to the proliferative compartment (basal cells) of well differentiated SCCs, whereas almost all the cells of

poorly differentiated, immunopositive SCCs (where poorly differentiated keratinocytes remain in the cell cycle), stained for p53. Increased expression of p53 in poorly differentiated cutaneous SCCs has previously been demonstrated (Gusterson et al, 1991; McGregor et al, 1992; Ro et al, 1992; Helander et al, 1993).

There is growing evidence that p53 functions as part of a cell cycle checkpoint control pathway (Kastan et al, 1991; El-Deiry et al, 1993 and 1994; Khanna & Lavin, 1993; Perry & Levine, 1993). It is therefore probable that multiple genes are involved in this pathway and that deregulation or inactivation of one (or more) of these genes may induce accumulation of p53 at critical times during the cell cycle. It is probable that tumours overexpressing wild type p53 have lost the growth regulatory response to the protein so are functionally similar to those expressing the mutant protein (Vojtesek & Lane, 1993). Therefore, this p53 accumulation may lead to increased levels of dysplasia and progression of cutaneous neoplasms. The complex cellular environment of chronic HPV infection and associated keratinocyte proliferation may provide multiple opportunities for gene mutations to result in the deregulation of p53. The identity of such genes and their roles in the accumulation of p53, and subsequent development of dysplasia and progression to skin cancer, remain to be established.

#### **4.3 THE RELATIONSHIP BETWEEN HPV AND p53 IN CUTANEOUS CARCINOGENESIS**

E6 oncoproteins from HPV types 16 and 18 can bind to and induce rapid degradation of wild type p53. From observations in anogenital cancers, it has been proposed that p53 inactivation occurs either by complexing of wild type p53 with such viral oncoproteins or, in the absence of virus, by mutational loss of gene function (Scheffner et al, 1990, 1991 and 1992; Werness et al, 1990; Crook et al, 1991a and b and 1992). This concept, however, remains controversial and other workers have failed to confirm these suggestions (Busby-Earle et al, 1993; Cooper et al, 1993). Evidence from this study suggests that, in contrast to cervical carcinogenesis, HPV contributes to skin



carcinogenesis through viral promotion of cell proliferation. The failure to demonstrate any relationship between the presence or absence of HPV DNA and accumulated p53 in dysplastic or frankly malignant skin lesions from RARs or ICPs is consistent with this viral promotion hypothesis. Furthermore, accumulated p53 was demonstrated in cutaneous neoplasia exhibiting HPV 16 DNA and p53 mutations were detected by SSCP analysis in both HPV positive and negative malignancies. Also in keeping with the suggestion that HPV do not contribute to cutaneous carcinogenesis by inactivating p53, is the recent demonstration that the E6 oncoprotein from skin associated HPV type 8 does not bind to p53, unlike its HPV 16 or 18 equivalents (Steger & Pfister, 1992). Therefore, HPV appear to contribute to cutaneous carcinogenesis by promoting keratinocyte proliferation, which is different to the mechanism of action of anogenital HPV types.

It is of interest that accumulated p53 was predominantly found in the basal layers of differentiated SCC. In papillomavirus infected epithelium, low levels of E6 and E7 proteins are found in the basal and suprabasal layers, increasing in the upper differentiated layers (Arends et al, 1990). Gusterson et al (1991) postulated that the low levels of E6 in the basal layers bind to and inactivate p53 resulting in an increased cell turnover and an increase in the pool of keratinocytes in which viral replication can take place. As cells differentiate, there is competition for binding of E6 between the p53 protein and viral DNA such that in the basal cells the equilibrium favours p53 with resultant proliferation while in the differentiated cells, with low endogenous levels of p53, DNA binding is favoured. The demonstration of accumulated p53 in basal cells indicates that this hypothesis is only likely if the E6 proteins from skin associated HPV types bind to and stabilise p53 without degradation, as is the case for SV40 large T antigen and adenovirus E1A. Although this does not appear to be true for cutaneous HPV types (Steger & Pfister, 1992), it would be of interest to carry out a double immunocytochemical assay, using antibodies directed to HPV E6 and p53, to

determine whether in those cases exhibiting HPV DNA and stabilised p53, HPV E6 is expressed in the p53 stained or unstained cells.

#### **4.4 THE ROLE OF UV RADIATION IN CUTANEOUS CARCINOGENESIS OF RARS**

Epidemiological studies strongly implicate UV light in the development of many epidermal tumours, including solar keratoses and SCCs (Blohme & Larko, 1984; Boyle et al, 1984; Yoshikawa et al, 1990; Marks, 1992). It has been discovered that patients with xeroderma pigmentosum (who are unable to repair UV-induced DNA photoproducts) have a 2000 fold increase in cutaneous SCCs. This suggests that one role of sunlight in the development of these malignancies is to induce genetic damage, thus acting as an initiating agent (Figure 4.1) (Brash et al, 1991; McGregor et al, 1991; Kraemer et al, 1984; Ziegler et al, 1993). Mutations due to direct absorption of UV light by DNA are predominantly C-T transitions at dipyrimidine sites. These include CC-TT double base mutations which are virtually pathognomonic of a UV based aetiology (Ananthaswamy & Pierceall, 1990; McGregor et al, 1991; Vogelstein & Kinzler, 1992). The recent demonstration of p53 mutations at dipyrimidine sites and the unique finding of CC-TT double base changes in epidermal tumours, but not in other common malignancies, provides further evidence that UV light may act as an initiating agent in cutaneous carcinogenesis (Brash et al, 1991; Ziegler et al, 1993; Campbell et al, 1993a). In this study, 5 of the 6 malignancies with mutated p53 developed on sun exposed body sites, implicating UV radiation in the genesis of these cancers. A role for UV was confirmed in one SCC that was found to have a UV specific (C-T) mutation at the codon 248 mutational hotspot of p53.

UV irradiation of mice induces a local and systemic immune response which is strongly associated with UV induced promotion of murine cutaneous neoplasms (Streilein, 1991). It has recently been demonstrated that UV radiation can suppress immune function in humans in a similar manner (Baadsgaard, 1991). Therefore, in RARs, UV



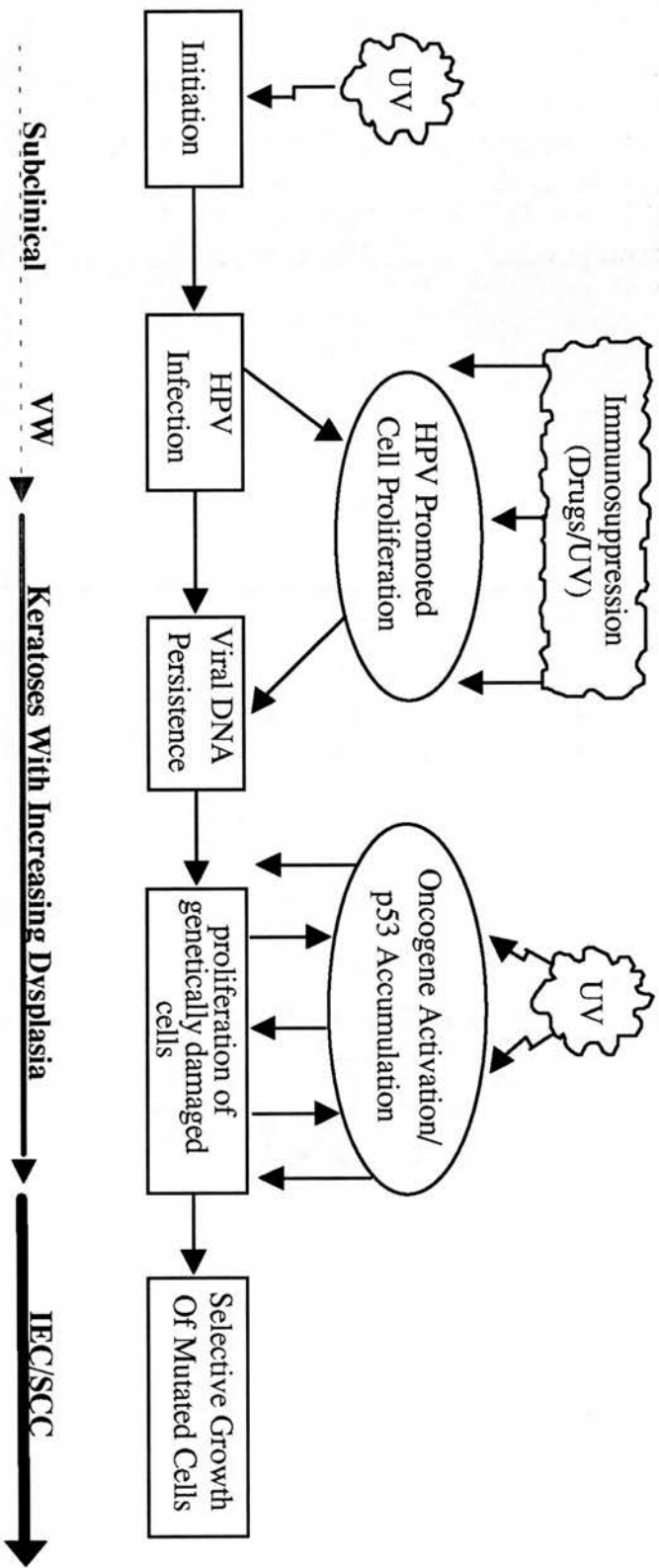
induced immunosuppression, in addition to transplant related immunosuppressive therapy, may allow chronic HPV infection and tumour formation and progression where rejection would otherwise occur with an intact immune response. This hypothesis is supported by the findings of this study that the majority of both viral warts and skin cancers developed on sun exposed body sites in RARs and that both types of lesion were more prevalent in patients with high sun exposure. Tilbrook et al (1989) have previously detected HPV DNA in papillomas and *in situ* and invasive SCCs of UV irradiated, hairless mice and suggested that these animals may be used as a model for viral carcinogenesis.

#### **4.5 A MODEL OF CUTANEOUS CARCINOGENESIS IN RARS**

On the basis of the data from this and other studies, a model of skin carcinogenesis may be proposed (Figure 4.1). Initially, UV radiation (or in a minority of cases, another carcinogen) induces an irreparable genetic lesion in an oncogene or tumour suppressor gene which results in the initiation of one or more epidermal keratinocytes. Exposure to high or chronic levels of UV radiation also results in local immunosuppression which, along with the effects of long term immunosuppressive therapy, allows persistent infection with any one of multiple different papillomavirus types. In a background of continuing sun exposure and immunosuppressive therapy, HPV stimulate high level proliferation of epidermal keratinocytes which expands the population of initiated cells and allows replication of damaged DNA without repair, generating further genetic damage. Eventually, mutations occur to a gene(s) involved in the regulation of p53, resulting in the accumulation and/or inactivation of wild type p53. Selection and clonal expansion of the cell population carrying inactivated p53 leads to increased levels of dysplasia and progression from dysplastic keratosis to IEC. Inactivation of p53 results in further uncontrolled cell proliferation and the

**Figure 4.1:-** Proposed scheme of HPV induced cutaneous carcinogenesis in RARs. HPV infection of epidermal keratinocytes causes cell proliferation, differentiation and the development of a viral wart. Chronic sun exposure and viral induced cell proliferation may lead to genetic damage and dysplasia of HPV infected cells within the viral wart. Alternatively, HPV infection of an epidermal keratinocyte carrying a UV induced initiating mutation results in proliferation of the genetically damaged cell and thus fixation of the genetic damage. Immunosuppressive therapy and the immunosuppressing effects of UV radiation allow viral DNA persistence and continued cell proliferation which may allow further genetic abnormalities and increasing levels of dysplasia. Genetic damage occurs in oncogenes or tumour suppressor genes and the lesion progresses towards malignancy.

**FIGURE 4.1:- PROPOSED MODEL OF CUTANEOUS CARCINOGENESIS OF RARS**



accumulation of more genetic lesions. One such lesion(s) causes further progression from *in situ* to invasive SCC.

This model of skin carcinogenesis is supported by the time scale of tumour development in RARs and ICPs. Although HPV infection may occur prior to or just months after transplantation, there is usually a time lapse of 5 to 10 years before the first skin cancer appears in transplant patients. Furthermore, in contrast to RARs where skin cancers may develop at a relatively early age, cutaneous SCCs are rarely observed in patients under seventy years of age in the immunocompetent population. It has been suggested that in ICPs, the initial genetic event inducing these lesions occurs 20 years prior to the development of the cancer (Kwa et al, 1992). These data suggest that a number of events may be required prior to the development of skin malignancies. Papillomavirus driven promotion of cell division in immunosuppressed patients may increase the chance of these genetic events occurring, which may explain the increased number and the early age of onset of skin tumours in RARs. In the absence of immunosuppressive therapy and papillomavirus infection, cutaneous tumours are probably promoted by UV radiation alone, which may explain the long time scale of skin cancer development in ICPs

Some features of this study may be explained by the above model of cutaneous carcinogenesis. The early acquisition of abnormal p53, through increased proliferation of epidermal keratinocytes, may account for the increased prevalence and extent of p53 immunostaining in dysplastic skin lesions, and the increased clinical rate of progression of such lesions, from RARs compared to those from ICPs (see Section 3.2 of the Discussion). In a background of deregulated cell proliferation, driven initially by HPV and subsequently by inactivation of p53, genetic damage may occur not only to the host cell genomic DNA but also to the infecting viral DNA. This may explain why in this study, a number of HPV were detected exhibiting deletions, amplifications and

mutations in their genome (see Section 2.3 of the Discussion). Under these circumstances, integration of viral DNA into the host genome may also occur as a random event, although, this appears to be rare in skin cancers.

The initiating genetic lesion in human skin cancers is presently unknown. In the mouse model of skin carcinogenesis, mutation of the *c-Ha-ras* oncogene is (in over 90% of tumours) strongly associated with the initiated state. However, the majority of reports suggest *ras* gene mutations are rare in human skin malignancies (Campbell et al, 1993c; Mukhtar & Bickers, 1993; Pierceall et al, 1991b). The presence of UV-associated, p53 mutations in skin cancers (Brash et al, 1991; Moles et al, 1993; Campbell et al, 1993a and b) suggests that p53 mutation may be one of the initial events in human skin carcinogenesis, however, this study indicated that this is only true in a minority of cases. The genetic event associated with malignant conversion of human skin cancers has also yet to be identified. In the mouse model of carcinogenesis, activation of the *fos* oncogene is associated with malignant conversion of keratinocytes (Greenhalgh et al, 1990). However, this gene has not yet been investigated in human skin cancers. Further investigations into early skin neoplasms (such as dysplastic keratoses) and cutaneous SCCs are required to determine the initiating events and the genetic events associated with malignant progression to invasive SCC in human skin carcinogenesis.

#### **4.6 CUTANEOUS CARCINOGENESIS IN INDIVIDUAL PATIENTS**

This study identified a sub-group of patients who appeared to be particularly susceptible to the development of skin neoplasia and consequently exhibited multiple skin cancers over the sample collection period (see Section 1 of the Discussion). Dysplastic and malignant cutaneous lesions from these patients were found to have an increased HPV prevalence compared to those from RARs with single or few skin cancers. HPV DNA was particularly prevalent in skin tumours from a 36 year old,



female RAR (patient 20) who, despite low levels of sun exposure, developed over 10 SCCs during the sample collection period. HPV DNA, including HPV 5 and 16, was detected in 70% of these lesions. This patient also developed multiple genital tract neoplasms and eventually died in 1993 of cervical cancer. HPV 5 and 16 have previously been found in some of these genital tract lesions (Alloub et al, 1989; Rudlinger et al, 1986). These data suggest that some RARs have an increased susceptibility to persistent HPV infection and consequently develop multiple carcinomas of the skin, and in some female RARs, the anogenital tract. This study is the first to demonstrate an association between the development of multiple skin cancers and an increased prevalence of HPV. Bouwes Bavinck et al (1993b) have recently demonstrated a strong link between HLA-Dr7, the absence of an antibody class switch (from IgM to IgG) in response to the L1 protein of HPV8 (or associated HPV types) and an increased incidence of skin cancer in RARs. In the present study, it is possible that some patients with multiple skin cancers may carry certain particular MHC class II subtypes that may affect the efficiency of mounting an effective immune response to HPV. This may exaggerate the effects of long term immunosuppressive therapy, resulting in chronic viral infection and skin cancer development. It has also been demonstrated that the immunosuppressive effects of UV radiation may be genetically determined (Yoshikawa et al, 1990). Therefore, an alternative explanation may be that RARs with multiple skin cancers have a genetic predisposition to the immunosuppressive effects of UV radiation and consequently develop multiple persistent HPV infections and skin neoplasms on UV exposed sites.

#### **4.7 FINAL CONCLUSIONS**

The findings of this study provide strong evidence that multiple different HPV types contribute to cutaneous carcinogenesis of RARs most likely by promotion of cell proliferation. At a practical level, these data challenge the necessity to systematically type HPV DNA found in cutaneous lesions from RARs. However, they highlight the



need for a sensitive assay to detect multiple different EV-associated and common cutaneous HPV types. These results also indicate that an effective vaccine against papillomavirus infection in transplant patients would have to be directed against viral epitopes which are conserved amongst all papillomavirus types, such as the L1 capsid protein. The finding that patients who develop multiple skin cancers have an increased prevalence of HPV infection is important. Further investigations are required to determine the reasons for this increased susceptibility to HPV infection in order to identify those transplant patients who may be at an increased risk of developing skin cancer. Resources may then be focussed on providing effective screening programs for these specific patients.

## **CHAPTER 6:-** **REFERENCES**

ABERER, W., SCHULER, G., STINGL, G., HONIGSMANN, H., WOLFF, K. (1981). Ultraviolet -light depletes surface-markers of Langerhans cells. *J. Invest. Dermatol.*, **76**, 202-210

ALLOUB, M.I., BARR, B.B.B, McLAREN, K.M., SMITH, I.W., BUNNEY, M.H. & SMART, G.E. (1989). Human papillomavirus and lower genital neoplasia in renal transplant patients. *Obstet. Gynecol.*, **68**, 251-258

ANANTHASWAMY, H.N., PIERCEALL, W.E. (1990). Molecular mechanisms of ultraviolet carcinogenesis. *Photochem. Photobiol.*, **52**, 1119-1136

ANDROPHY, E.J., LOWY, D.R., SCHILLER, J.T. (1987). Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. *Nature*, **325**, 70-73

ARENDS, M.J., WYLLIE, A.H., BIRD, C.C. (1990). Papillomaviruses and human cancer. *Hum Pathol.* **21**, 686-698

ARENDS, M.J., DONALDSON, Y.K., DUVALL, E., WYLLIE, A.H., BIRD, C.C. (1991). HPV in full thickness cervical biopsies: high prevalence in CIN 2 and CIN 3 detected by a sensitive PCR assay. *J. Pathol.*, **165**, 301-309

ARENDS, M.J., DONALDSON, Y.K., DUVALL, E., WYLLIE, A.H., BIRD, C.C. (1993). Human papillomavirus type 18 associates with more advanced cervical neoplasia than human papillomavirus type 16. *Hum Pathol.* **24**, 432-437.

ARENDS, M.J., MCGREGOR, A.H., WYLLIE, A.H. (1994). Apoptosis is inversely related to necrosis and determines net growth in tumours bearing constitutively expressed *myc*, *ras* and HPV oncogenes. *Am. J. Pathol.*, **144**, 1045-1057

ARENDS, M.J., HARRISON, D.J. (1994). Apoptosis: molecular aspects and pathological perspective. in *Molecular Biology In Histopathology* (J. Crocker Editor). pp151-169

ASHINOFF, R., LI, J.J., JACOBSON, M., FRIEDMAN-KEIN, A.E., GERONEMUS, R.G. (1991). Detection of human papillomavirus DNA in squamous cell carcinoma of the nail bed and finger determined by polymerase chain reaction. *Arch. Dermatol.*, **127**, 1813-1818

BAADSGAARD, O. (1991). In vivo ultraviolet radiation of human skin results in profound perturbation of the immune system. *Arch. Dermatol.*, **127**, 99-109

BAAS, I.O., MULDER, J-W. R., OFFERHAUS, G.J.A., VOGELSTEIN, B., HAMILTON, S.R. (1994). An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J. Pathol.*, **172**, 5-12

- BAKER, S.J., FEARON, E.R., NIGRO, J.M., HAMILTON, S.R., PREISINGER, A.C., JESSUP, J.M., VAN TUINEN, P., LEDBETTER, D.H., BARKER, D.F., NAKAMURA, Y., WHITE, R., VOGELSTEIN, B. (1989). Chromosome 17p deletions and p53 gene mutations in colorectal carcinomas. *Science*, **249**, 912-915
- BAKER, C.C. (1987). in *The Papovaviridae, the papillomaviruses*. (Salzman, N.P and Howley, P.M Eds). pp321-385. Plenum Press, New York
- BAKER, S.J., PREISINGER, A.C., JESSUP, J.M., PARASKEVA, C., MARHOWITZ, S., WILSON, J.K.V., HAMILTON, S., VOGELSTEIN, B. (1990a). P53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumourigenesis. *Cancer Res.*, **50**, 7717-7722.
- BAKER, S.J., MARKOWITZ, S., FEARON, E.R., WILLSON, J.K.V., VOGELSTEIN, B. (1990b). Suppression of human colorectal carcinoma cell growth by wild type p53. *Science*, **249**, 912-915
- BALMAIN, A., RAMSDEN, M., BOWDEN, G.T., SMITH, J. (1984). Activation of the mouse cellular Harvey-ras gene in chemically induced, benign skin papillomas. *Nature*, **307**, 658-660
- BAND, V., DE CAPRIO, J.A., DELMOLINO, L., KULESA, V., SAGER, R. (1991). Loss of p53 protein in human mammary epithelial cells. *J. Virol.*, **65**, 6671-6676
- BANKS, L., MATLASHEWSKI, G. & CRAWFORD, L. (1986). Isolation of human-p53-specific monoclonal antibodies and their use in the studies of human p53 expression. *Eur. J. Biochem*, **159**, 529-534
- BARBOSA, M.S., EDMONDS, C., FISHER, C., SCHILLER, J.T., LOWRY, D.R., VOUSDEN, K.H. (1990). The region of the HPV E7 oncoprotein homologous to adenovirus E1A and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. *EMBO. J.*, **9**, 153-160
- BARBOSA, M.S., VASS, W.C., LOWRY, D.R., SCHILLER, J.T. (1991). In vitro biological activities of the E6 and E7 genes vary among HPVs of different oncogenic potential. *J. Virol.*, **65**, 292-298
- BARNES, D.M., HANBY, A.M., GILLET, C.E., MOHAMMED, S., HODGSON, S., BOBROW, L.G., LEIGH, I.M., PURKIS, T., MACGEOCH, C., SPURR, N.K., BARTEK, J., VOJTESEK, B., PICKSEY, S.M., LANE, D.P. (1992). Abnormal expression of wild type p53 protein in normal cells of a cancer family patient. *Lancet*, **340**, 259-263
- BARNES, D.M., DUBLIN, E.A., FISHER, C.J., LEVISON, D.A., MILLIS, R.R. (1993). Immunohistochemical detection of p53 in mammary carcinomas: an important new independent indicator of prognosis? *Hum. Pathol.*, **24**, 469-476

- BARNETT, N., MAK, H., WINKELSTEIN, J.A. (1983). Extensive verrucosis in primary immunodeficiency diseases. *Arch. Dermatol*, **119**, 5-7
- BARR, B.B.B, BENTON, E.C., McLAREN, K.M., BUNNEY, M.H., SMITH, I.W., BLESSING, K. & HUNTER, J.A.A. (1989). Human papilloma virus infection and skin cancer in renal allograft recipients. *Lancet*, **I**, 124-129
- BARRETT, W.L., FIRST, M.R., ARON, B.S., PENN, I. (1993). Clinical course of malignancies in renal transplant recipients. *Cancer*, **72**, 2186-2189
- BARTEK, J., IGGO, R., GANNON, J., LANE, D.P. (1990a). Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene*, **5**, 893-899
- BARTEK, J., BARTKOVA, J., VOJTESEK, B., STASKOVA, Z., REJTHAR, A., KOVARIK, J., MIDGLEY, C.A., GANNON, J.V., LANE, D.P. (1990b). Patterns of expression of the p53 tumour suppressor in human breast tissues and tumours *in situ* and *in vitro*. *Int. J. Cancer*, **46**, 839-844
- BARTEK, J., BARTKOVA, J., VOJTESEK, B., STASKOVA, Z., LUCAS, J., REJTHAR, A., KOVARIK, J., MIDGLEY, C.A., GANNON, J.V., LANE, D.P. (1991). Abberant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. *Oncogene*, **6**, 1699-1703
- BEDELL, M.A., HUDSON, J.B., GOLUB, T.R., TURK, M.F., HOSKEN, M., WILBANHS, C.D., LAIMINS, L.A. (1991). Amplification of HPV genomes in vitro is dependant on epithelial differentiation. *J. Virol.*, **65**, 2254-2260
- BENCHIMOL, S., PIM, D., CRAWFORD, L. (1982). Radioimmunoassay of the cellular protein p53 in mouse and human cell lines. *EMBO. J.*, **1**, 1055-1062
- BENDORI, R., RESNITZKI, O., KIMCHI, A. (1987). Changes in p53 messenger-RNA expression during terminal differentiation of murine erythroleukemia cells. *Virology*, **161**, 607-611
- BENNET, W.P., HOLLSTEIN, M.C., HSU, I-C., SIDRANSKY, D., LANE, D.P., VOGELSTEIN, B., HARRIS, C.C. (1992). Mutational spectra and immunohistochemical analysis of p53 in human cancers. *Chest*, **101**, 19s-21s
- BENTON, C., SHAHIDULLAH, H., HUNTER, J.A.A. (1992). Human papillomavirus in the immunosuppressed. *Papillomavirus Report*, **3**, 23-26
- BERNARD, B.A., BAILLY, C., LENOIR, M-C., DARMAN, M., THIERRY, F., YANIV, M. (1989). The human papillomavirus type 18 (HPV 18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. *J. Virol.*, **63**, 4317-4324



- BIRKELAND, S.A. (1983). Malignant tumours in renal transplant patients. *Cancer*, **51**, 1571-1575
- BISCHOFF, J.R., FREIDMAN, P.N., MARSHAK, D.R., PRIVES, C., BEACH, D. (1990a). Human p53 is phosphorylated by p60 cdc2 and cyclin-B-cdc2. *Proc. Natl. Acad. Sci. USA.*, **87**, 4766-4770
- BISCHOFF, J.P., YIM, S.O., PATHAK, S., GRANT, G., SICILANO, M.J., GIOVANELLA, B.C., STRONG, L.C., TAINSKY, M.A. (1990b). Spontaneous abnormalities in normal fibroblasts from patients with Li-fraumeni cancer syndrome: aneuploidy and immortalisation. *Cancer Res.*, **50**, 7979-7984
- BLESSING, K., McLAREN, K.M., BENTON, E.C., BARR, B.B., BUNNEY, M.H., SMITH, I.W. & BEVERIDGE, G.W. (1989). Histopathology of skin lesions in renal allograft recipients-an assessment of viral features and dysplasia. *Histopathology*, **14**, 129-139
- BLOHME, I. and LARKO, O. (1984). Pre-malignant and malignant skin lesions in renal transplant patients. *Transplantation*, **37**, 165-167
- BODNER, S.M., MINNA, J.D., JENSEN, S.M., D'AMICO, D., CARBONE, D., MITSUDOMI, T., FEDORKO, J., BUCHHAGEN, D.L., NAU, M.M., GAZDAR, A.F. & LINNOILA, R.I. (1992). Expression of mutant p53 proteins in lung cancer correlates with the class of p53 gene mutation. *Oncogene*, **7**, 743-749
- BOSHART, M., GISSMANN, L., IKENBERG, H., KLEINHEINZ, A., SCHEURLIN, W., ZUR HAUSEN, H. (1984). New type of papillomavirus DNA, it's prevalence in genital cancer biopsies and in cell-lines derived from cervical cancer. *EMBO. J.*, **3**, 1151-1157
- BOUWES BAVINCK, J.N., VERMEER, B.J., VAN DER WOUDE, F.J., VANDENBROUCHE, J.P., SCHREUDER, G.M.Th., THOROGOOD, J., PERSIJN, G.G., CLAAS, F.H.J. (1991a). Relation between skin cancer and HLA antigens in renal-transplant recipients. *New Engl. J. Med.*, **325**, 843-848
- BOUWES BAVINCK, J.N., KOOTE, A.M.M., VAN DER WOUDE, F.J., VANDENBROUCHE, J.P., VERMEER, B.J., CLAAS, F.H.J. (1991b). On a possible protective effect of HLA -All against skin cancer and keratotic skin lesions in renal transplant recipients. *J. Invest. Dermatol.*, **97**, 269-272
- BOUWES BAVINCK, J.N., DE BOER, A., VERMEER, B.J., HARTEVELT, M.M., VAN DER WOUDE, F.J., CLAAS, F.H.J., WOLTERBEEK, R., VANDENBROUCKE, J.P. (1993a). Sunlight, keratotic skin lesions and skin cancer in renal transplant recipients. *B. J. Dermatol.*, **129**, 242-249

- BOUWES BAVINCK, J.N., GISSMANN, L., CLAAS, F.H.J., VAN DER WOUDE, F.J., PERSIJN, G.G., SCHEGGET, J.T., VERMEER, B.J., JOCHMUS, I., MULLER, M., STEYER, G., GEBERT, S., PFISTER, H. (1993b). Relation between skin cancer, humoral responses to human papillomaviruses and HLA class II molecules in renal transplant recipients. *J. Immunol.*, **151**, 1579-1586
- BOWDEN, T., KRIEG, P. (1991). Differential gene expression during multistage carcinogenesis. *Environ. Health Prospect.*, **93**, 51-56
- BOYLE, J., MACKIE, R.M., BRIGGS, J.D., JUNOR, B.J.R. & AITCHISON, T.C. (1984). Cancer, warts and sunshine in renal transplant patients. A case control study. *Lancet*, **I**, 702-705
- BRADFORD, C.R., HOFFMAN, H.T., WOLF, G.T., COREY, T.E., BAKER, S.R., McCLATCHEY, K.D. (1990). Squamous cell carcinoma of the head and neck in organ transplant recipients: possible role of oncogenic viruses. *Laryngoscope*, **100**, 190-194
- BRASH, D.E., RUDOLPH, J.A., SIMON, J.A., LIN, A., McKENNA, G.J., BADEN, H.P., HALPERIN, A.J. & PONTEN, J. (1991). A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc. Natl. Acad. Sci. USA*, **88**, 10124-10128
- BRAVERMAN, I.M. (1991). Bowens disease and internal cancer (editorial). *JAMA*. **266**, 842-843
- BREITURD, F., CROISSANT, O., ORTH, G. (1987). Expression of human papillomavirus type-1 E4 gene products in warts. *Cancer Cells*, **5**, 115-122
- BRESSAC, B., KEW, M., WANDS, J., OZTURK, M. (1991). Selective G to T mutations of the p53 gene in hepatocellular carcinoma from southern Africa. *Nature*, **350**, 429-431
- BRIGGAMAN, R.A., WHEELER, C.E. (1979). Immunology of human warts. *J. Am. Acad. Dermatol.*, **1**, 297-304
- BRINTON, L.A., NASCA, P.C., MALLIN, K., BAPTISTE, M.S., WILBANKS, G.D., RICHART, R.M. (1990). Case control study of cancer of the vulva. *Obstetrics and Gynecology*, **75**, 859-866
- BRINTON, L.A. (1992). Epidemiology of cervical cancer-overview. In the Epidemiology of Human Papillomavirus And Cervical Cancer (N Munoz, F.X. Bosch, K.U. Shah, A. Mehews. Eds). Oxford University Press. pp3-23
- BROWNING, M.J., BODMER, W.F. (1992). MHC antigens and cancer: implications for T cell surveillance. *Current Opinion in Immunol.*, **4**, 613-618

- BUNNEY, M.H., BENTON, E.C., BARR, B.B., SMITH, I.W., ANDERSON, J.L., HUNTER, J.A.A. (1990). The prevalence of skin disorders in renal allograft recipients receiving cyclosporin A compared with those receiving Azathioprine. *Nephrol. Dial. Transplant.*, **5**, 379-382
- BUNNEY, M.H., BENTON, C., CUBIE, H. (1992). Warts in the immunodeficient. *in Viral Warts, Biology And Treatment* (Second Edition). Oxford university press. pp65-72
- BURNS, P.A., BREMNER, R., BALMAIN, A. (1991). Genetic changes during mouse skin tumourigenesis. *Environ. Health Prospect.*, **93**, 41-44
- BURNS, J.E., BAIRD, L.J., CLARK, P.A., BURNS, K., EDINGTON, K., CHAPMAN, C., MITCHELL, C.R., ROBERTSON, G., SOUTAR, D. & PARKINSON, E.K. (1993). Gene mutations and increased levels of p53 protein in human squamous cell carcinomas and their cell lines. *Br. J. Cancer*, **67**, 1274-1284
- BUSBY-EARLE, R.M.C., STEEL, C.M., BIRD, C.C. (1993). Cervical carcinoma: low frequency of allele loss at loci implicated in other common malignancies. *Br. J. Cancer*, **67**, 71-75
- CAMPBELL, C., QUINN, A.G., RO, Y-S., ANGUS, B. & REES, J.L. (1993a). p53 mutations are a common and early event which precede tumour invasion in squamous cell neoplasia of the skin. *J. Invest. Dermatol.*, **100**, 746-748
- CAMPBELL, C., QUINN, A.G., ANGUS, B. & REES, J.L. (1993b). The relation between p53 mutation and p53 immunostaining in non-melanoma skin cancer. *Br. J. Dermatol.*, **129**, 235-241
- CAMPBELL, C., QUINN, A.G., REES, J.L. (1993c). Codon 12 Harvey-ras mutations are rare events in non-melanoma human skin cancer. *B. J. Dermatol.*, **128**, 111-114
- CAMPO, M.S., MOAR, M.H., SARTIRANA, M.C., KENNEDY, I.M., JARRETT, W.F. (1985). The presence of Bovine papillomavirus type 4 DNA is not required for the progression to or maintenance of the malignant state in cancers of the alimentary tract in cattle. *EMBO. J.*, **4**, 1819-1825
- CARDER, P., WYLLIE, A.H., PURDIE, C.A., MORRIS, R.G., WHITE, S., PIRIS, J., BIRD, C.C. (1993). Stabilised p53 facilitates aneuploid clonal divergence in colorectal cancer. *Oncogene*, **8**, 1397-1401
- CARON DE FROMENTEL, O., MAY-LEVIN, F., MOURIESSE, H., LEMERLE, O., CHANDRASEHARAN, K., MAY, P. (1987). Presence of circulating antibodies against cellular protein p53 in a notable proportion of children with B-cell lymphoma. *Int. J. Cancer*, **39**, 185-189

- CATTORETTI, G., RILKE, F., ANDREOLA, S., D'AMATO, L., DELIA. (1988). p53 expression in breast cancer. *Int. J. Cancer*, **41**, 178-183
- CHANG, F. (1990). Role of papillomaviruses. *J. Clin. Pathol.*, **43**, 269-276
- CHANG, F., SYRJANEN, S., TERVAHAVTA, A., SYRJANEN, K. (1993). Tumourigenesis associated with the P53 tumor suppresser gene. *Br. J. Cancer*, **68**, 653-661
- CHELLAPPAN, S., KRAUS, V., KROGER, B., MUNGER, K., HOWLEY, P.M., PHELPS, W.C., NEVINS, J.R. (1992). Adenovirus E1A, simian virus 40 tumour antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. *Proc. Natl. Acad. Sci. USA*, **89**, 4549-4553
- CHEN, P.M., CHEN, Y.M., BOOKSTEIN, R., LEE, W.H. (1990). Genetic mechanisms of tumour suppression by the human p53 gene. *Science*, **250**, 1576-1580
- CHEN, S.L., MOUNTS, P. (1990). Transforming activity of E5a protein of human papillomavirus type 6 in NIH 3T3 and C127 cells. *J. Virol*, **64**, 3226-3233
- CHIBA, I., TAKAHASHI, T., NAU, M.M., DAMICO, D., CURIEL, D.T., MITSUDOMI, T., SLAMON, D.J., HOLMES, E.C., MINNA, J.D. (1990). Mutations in the p53 gene are frequent in primary, resected non-small cell lung cancer. *Oncogene*, **5**, 1603-1610
- CHIN, M.T., HIROCHIKA, R., HIROCHIKA, H. (1988). Regulation of human papillomavirus 11 enhancer and E6 promotor by activating and repressing proteins from the E2 ORF : functional and biochemical studies. *J. Virol.*, **62**, 2994-3002
- CHO, Y., GORINA, S., JEFFREY, P., PAVLETICH, N.P. (1994). Crystal structure of a p53 tumour suppressor DNA complex - understanding tumourigenic mutations. *Science*, **265**, 346-355
- CHUMAKOV, A.M., MILLAR, C.W., CHEN, D.L., KOEFFLER, H.P. (1993). Analysis of p53 transactivation through high affinity binding sites. *Oncogene*, **8**, 3005-3011
- CIECHANOVER, A., DIGIUSEPPE, J.A., BERCOVICH, B., ORIAN, A., RICHTER, J.D., SCHWARTZ, A.L., BRODEUR, G.M. (1991). Degradation of nuclear oncoproteins by the ubiquitin system in vitro. *Proc. Natl. Acad. Sci. USA*, **88**, 139-143
- CLARKE, A.R., PURDIE, C.A., HARRISON, D.J., MORRIS, R.G., BIRD, C.C., HOOPER, M.L. & WYLLIE, A.H. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, **362**, 849-852



- CONRAD, M., BUBB, V.J., SCHLEGEL, R. (1993). The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-Kilodalton pore-forming protein. *J. Virol.*, **67**, 6170-6178
- COOPER, K., HERRINGTON, C.S., EVANS, M.F., GATTER, K.C., McGEE, J.O.D. (1993). p53 antigen in cervical condylomata, intraepithelial neoplasia and carcinoma: relationship to HPV infection and integration. *J. Pathol.*, **171**, 27-34
- CRIFE, T.P., HAUGEN, T.H., TURK, J.P., TABATABAI, F., SCHMID, P.G., DURST, M., GISSMANN, L., ROMAN, A., TUREK, L.P. (1987). Transcriptional regulation of the human papillomavirus-16 E6-E7 promotor by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products: implications for cervical carcinogenesis. *EMBO. J.*, **6**, 3745-3753
- CRIPPS, K.J., PURDIE, C.A., CARDER, P.J., WHITE, S., KOMINE, K., BIRD, C.C., WYLLIE, A.H. (1994). A study of stabilisation of p53 protein versus point mutation in colorectal carcinoma. *Oncogene*, **9**, 2739-2743
- CROOK, T., WREDE, D., TIDY, J.A., SCHOLFIELD, J., CRAWFORD, L. & VOUSDEN, K.H. (1991a). Status of c-myc, p53 and retinoblastoma genes in human papillomavirus positive and negative squamous cell carcinomas of the anus. *Oncogene*, **6**, 1251-1257
- CROOK, T., TIDY, J.A., VOUSDEN, K.H. (1991b). Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and transactivation. *Cell*, **67**, 547-556
- CROOK, T., FISHER, C. and VOUSDEN, K.H., (1991c). Modulation of immortalizing properties of human papillomavirus type 16 E7 by P53 expression. *J. Virol.*, **65**, 505-510.
- CROOK, T., WREDE, D., TIDY, J.A., MASON, W.P., EVANS, D.J., VOUSDEN, K.H. (1992). Clonal p53 mutation in primary cervical cancer: association with human papillomavirus negative tumours. *Lancet*, **339**, 1070-1073
- CRUZ, P.D., BERGSTRESSER, P.R. (1989). Ultraviolet radiation, Langerhan's cells and skin cancer. *Arch. Dermatol.*, **125**, 975-978
- CULLEN, A.P., REID, R., CAMPION, M., LORINCZ, A.T. (1991). Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasms. *J. Virol*, **65**, 606-612
- CUNNINGHAM, J., LUST, J.A., SCHAID, D.J., BERN, G.D., CARPENTER, H.A., RIZZA, E., KOVACH, J.S., THIBODEAU, S.N. (1992). Expression of p53 and 17p allelic loss in colorectal carcinoma. *Cancer Res.*, **52**, 1974-1980



- DANOS, O., KATINKA, M., YANIV, M. (1982). Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among Papovaviridae. *EMBO J.* **1**, 231-236
- DANOS, O., ENGL, L.W., CHEN, E.Y., YANIV, M., HOWLEY, P.M. (1983). A comparative analysis of the human type 1a and bovine type 1 papillomavirus genomes. *J. Virol.*, **46**, 557-566
- DAVIES, R., HICKS, R., CROOK, T., MORRIS, J., VOUSDEN, K.H. (1993). HPV16 E7 associates with histone H1 kinase activity and p107 through sequences necessary for transformation. *J. Virol.*, **67**, 2521-2528
- DE VILLIERS, E.M. (1989). Heterogeneity of the human papillomavirus group. *J. Virol.*, **63**, 4898-4903
- DEAU, M.C., FAVRE, M., ORTH, G. (1991). Genetic heterogeneity among human papillomaviruses (HPV) associated with epidermodysplasia verruciformis: evidence for multiple allelic forms of HPV 5 and HPV8 E6 genes. *Virology*, **184**, 492-503
- DEBBAS, M., WHITE, E. (1993). Wild type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes and Dev.*, **71**, 546-554
- DEFFIE, A., WU, H., REINKE, V., LOZANO, G. (1993). The tumour suppressor p53 regulates it's own transcription. *Mol. Cell. Biol.*, **13**, 3415-3423
- DELATTRE, O., OLSCHWANG, S., LAW, D.J., MELOT, T., REMVIKOS, Y., SALMON, R.J., SASTRE, X., VALIDIRE, P., FEINBERG, A.P., THOMAS, G. (1989). Multiple genetic alterations in distal and proximal colorectal cancer. *Lancet*, 353-356
- DIPAULO, J.A., WOODWORTH, C.D., POPESOU, N.C. (1989). Induction of human cervical squamous cell carcinoma by sequential transfection by HPV 16 DNA and viral Harvey ras. *Oncogene*, **4**, 395-399
- DILLER, L., KASSEL, J., NELSON, C.E., GRYHA, M.A., LITWAK, G., GEBHARDT, M., BRESSAC, B., OZTURK, M., BAKER, S.J., VOGELSTEIN, B., FREIND, S.H. (1990). p53 functions as a cell cycle control protein in osteosarcomas. *Mol. Cell Biol.*, **10**, 5772-5781
- DIPPOLD, W.G., JAY, G., DELEO, A.B., KHOURY, G.K., OLD, L.J. (1981). p53 transformation related protein : detection by monoclonal Ab in mouse and human cells. *Proc. Natl. Acad. Sci. USA.*, **78**, 1695-1699
- DITTMER, D., PALI, S., ZAMBETTI, G., CHU, S., TERESKY, A.K., MOORE, M., FINLAY, C. & LEVINE, A.J. (1993). Gain of function mutations in P53. *Nature Genetics*, **4**, 42-45.

- DODSON, J.M., DESPAIN, J., HEWETT, J.E., CLARK, D.P. (1991). Malignant potential of actinic keratosis and the controversy over treatment. *Arch. Dermatol.*, **127**, 1029-1031
- DONEHOWER, L.A., HARVEY, M., SLAGLE, B.L., MCARTHUR, M.J., MONTGOMERY, C.A., BITEL, J.S., BRADLEY, A. (1992). Mice deficient for P53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, **356**, 215-221.
- DONEHOWER, L.A., BRADLEY, A. (1993). The tumor suppressor p53. *Biochemica et Biophysica acta*, **1155**, 181-205
- DOORBAR, J., CAMPBELL, D., GROIND, R.J.A., GALLIMORE, P.H. (1988). Identification of the human papilloma virus-1a E4 gene products. *EMBO. J.*, **5**, 355-362
- DOORBAR, J., ELY, S., STERLING, J., McLEAN, C., CRAWFORD, L. (1991). Specific interaction between HPV 16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature*, **352**, 824-827
- DOTTO, G.P., O'CONNELL, J., PATSHAN, G., CONTI, C., ARIZA, A., SLAGA, T.J. (1988). Malignant progression of papilloma-derived keratinocytes: differential effects of the ras, neu and p53 oncogenes. *Mol. Carcinog.*, **1**, 171-179
- DUNN, J.M., HASTRICH, D.J., NEWCOMB, P., WEBB, J.C.J., MAITLAND, N.J., FARNDON, J.R. (1993). Correlation between p53 mutation and antibody staining in breast-carcinoma. *Br. J. Surgery*, **80**, 1410-1412
- DURST, M., GISSMANN, L., IKENBERG, H., ZUR HAUSEN, H. (1983). A papillomavirus DNA from a cervical-carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. USA*, **80**, 3812-3815
- DYALL-SMITH, D., TROWELL, H., MARK, A., DYALL-SMITH, M. (1991). Cutaneous squamous cell carcinomas and papillomaviruses in renal transplant recipients: A clinical and molecular biological study. *J. Dermatol. Sci.*, **2**, 139-146
- DYSON, N., HOWLEY, P.M., MUNGER, K., HARLOW, E. (1989). The human papilloma virus- 16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science*, **243**, 934-936.
- ECCLES, D.M., BRETT, L., LESSELLS, A., GRUBER, L., LANE, D., STEEL, C.M., LEONARD, R.C.F. (1992). Overexpression of the p53 protein and allele loss at 17p13 in ovarian carcinoma. *Br. J. Cancer*, **65**, 40-44
- EL-DEIRY, W.S., KERN, S.E., PIETENPOL, J.A., ZINZLER, K.W., VOGELSTEIN, B. (1992). Definition of a consensus binding site for p53. *Nature Genetics*, **1**, 45-49

EL-DEIRY, W.S., TOKINO, T., VELCULESCU, V.E., LEVY, D.B., PARSONS, R., TRENT, J.M., LIN, D., MERCER, W.E., KINZLER, K.W., VOGELSTEIN, B. (1993). WAF1, a potential mediator of p53 tumour suppression. *Cell*, **75**, 817-825

EL-DEIRY, W.S., HARPER, J.W., O'CONNER, P.M., VELCULESCU, V.E., CANMAN, C.E., JACKMAN, J., PIETENPOL, J.A., BURRELL, M., HILL, D.F., WANG, Y., WIMAN, K.G., MERCER, W.E., KASTAN, M.B., KOHN, K.W., ELLEDGE, S.J., KINZLER, W., VOGELSTEIN, B. (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.*, **54**, 1169-1174

ELIEZRI, Y.D., SILVERSTEIN, S.J., NUOVO, G.J. (1990). Occurrence of human papillomavirus type 16 DNA in cutaneous squamous and basal cell neoplasms. *J. Am Acad Dermatol.*, **23**, 836-842.

ELIYAHU, D., MICHALOVITZ, D., ELIYAHU, S., PINHASI-KIMHI, O., OREN, M. (1989). Wild type p53 can inhibit oncogene mediated focus formation. *Proc. Natl. Acad. Sci. USA.*, **86**, 8763-8767

ENSSER, A., PFISTER, H. (1990). Epidermodysplasia verruciformis associated human papillomavirus present a subgenus-specific organization of the regulatory genome region. *Nucleic Acids Res.*, **18**, 3919-3922

EUVRARD, S., CHARDONNET, Y., POUTEIL-NOBLE, C., KANITAKIS, J., CHIGNOL, M.C., THIVOLET, J., TOURAINE, J.L. (1993). Association of skin malignancies with various multiple carcinogenic and non carcinogenic human papillomaviruses in renal transplant patients. *Cancer*, **72**, 2198-2206

FARMER, G., BARGONETTI, J., ZHU, H., FRIEDMAN, P., PRYWES, R., PRIVES, C. (1992). Wild type p53 activates transcription in vitro. *Nature*, **358**, 83-86

FEINSTEIN, E., GALE, R.P., REED, J., CANAANI, E., (1992), Expression of the normal p53 gene induces differentiation of K562 cells. *Oncogene*, **7**, 1853-1857

FINDLAY, C.A., HINDS, P.W., TAN, T.H., ELIYAHU, D., OREN, M.E., LEVINE, A.J. (1988). Activating mutations for transformation by p53 produce a gene product that forms a hsc70-p53 complex with an altered half life. *Mol. Cell Biol.*, **8**, 531-539

FINDLAY, C.A., HINDS, P.W., LEVINE, A.J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. *Cell*, **57**, 1083-1093

FINDLAY, C.A. (1993). The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth. *Mol. Cell. Biol.*, **13**, 301-306

- FISHER, M.S., KRIPKE, M.L. (1977). Systemic alteration induced in mice by ultraviolet light irradiation and its relationship to ultraviolet light carcinogenesis. *Proc. Natl. Acad. Sci. USA.*, **74**, 1688-1692
- FRENTZ, G., MOLLER, U. (1983). Clonal heterogeneity in curetted human epidermal cancers and precancers analyzed by flow-cytometry and compared with histology. *Br. J. Dermatol.*, **109**, 173-181
- FRIERSON, H.F., DEUTSCH, B.D., LEVINE, P.A. (1988). Clinicopathologic features of cutaneous squamous cell carcinoma of the head and neck in patients with chronic lymphocytic leukemia / small lymphocytic lymphoma. *Hum. Pathol.*, **19**, 1397-1402
- FUCHS, P.G., IFTNER, T., WENINGER, J., PFISTER, H. (1986). Epidermodysplasia verruciformis-associated human papillomavirus 8: genomic sequence and comparative analysis. *J. Virol.* **58**, 626-634
- FUCHS, P.G., PFISTER, H. (1990). Papillomaviruses in epidermodysplasia verruciformis. *Papillomavirus Report*, **1**, 1-4
- FUJIKI, H., SUGANUMA, M., YOSHIZAWA, S., WINYAR, B., SUGIMURA, T. (1991). Mechanisms of action of the okadaic acid class of tumour promoters on mouse skin. *Environ. Health Prospect.*, **93**, 211-214
- FUKUSHIMA, M., YAMAKAWA, Y., SHIMANO, S., HASHIMOTO, M., SAWADA, Y., FUJINAGA, K. (1990). The physical state of human papillomavirus type 16 DNA in cervical intraepithelial neoplasia. *Cancer*, **66**, 2155-2161
- FUNK, W.D., PAK, D.T., KARAS, R.H., WRIGHT, W.E., SHAY, J.W. (1992). A transcriptionally active, DNA-binding site for human p53 protein complexes. *Mol. Cell. Biol.*, **12**, 2866-2871
- GANNON, J.V., GREAVES, R., IGGO, R. & LANE, D.P. (1990). Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO. J.*, **9**, 1595-1602
- GASSENMAIER, A., LANNEL, M., PFISTER, H. (1984). Molecular cloning and characterization of the DNAs of human papillomavirus 19, 20 and 25 from a patient with epidermodysplasia verruciformis. *J. Virol.*, **52**, 1019-1023
- GIRI, I., DANOS, O. (1986). Papillomavirus genomes: from sequence data to biological properties. *Trends In Genetics*, **2**, 227-232
- GISSMANN, L., DIEHL, V., SCHULTZ-COULON, H.J., ZUR HAUSEN, H. (1982). Molecular cloning and characterisation of human papillomavirus DNA derived from a laryngeal papilloma. *J. Virol.*, **44**, 393-400



- GLOVER, M.T., BODMER, J., KENNEDY, L.J., BROWN, J., NAVARRETE, C., KWAN, J.T.C., LEIGH, I.M. (1993). HLA antigen frequencies in renal transplant recipients and non-immunosuppressed patients with non-melanoma skin cancer. *Eur. J. Cancer*, **29A**, 520-524
- GOLDSTEIN, D.J., FINBOW, M.E., ANDRESSON, T., MCLEAN, P., SMITH, K., BUBB, V., SCHLEGEL, R. (1991). The bovine papillomavirus E5 oncoprotein binds to the 16 kilodalton component of vacuolar H<sup>+</sup>-ATPases. *Nature*, **352**, 347-349
- GOLDSTEIN, D.J., ANDRESSON, T., SPARKOWSKI, J.J., SCHLEGEL, R. (1992). The BPV-1 E5 protein, the 16kD membrane pore-forming protein, and the PDGF receptor exist in a complex which is dependent upon hydrophobic transmembrane interactions. *EMBO. J.*, **11**, 4851-4859
- GREENHALGH, D.A., WELTY, D.J., PLAYER, A. & YUSPA, S.H. (1990). Two oncogenes, v-fos and v-ras cooperate to convert normal keratinocytes to squamous cell carcinoma. *Proc. Natl. Acad. Sci. USA.*, **87**, 643-647
- GROSS, G., ELLINGER, K., ROUSSAKI, A., FUCHS, P.G., PETER, H.H., PFISTER, H. (1988). Epidermodysplasia verruciformis in a patient with Hodgkins disease-characterisation of a new papillomavirus type and interferon treatment. *J. Invest. Dermatol.*, **91**, 43-48
- GRUBER, S.A., SKJEI, K.L., SOUTHERN, R.B., ROBISON, L., TZARDIS, P., MOSS, A., GILLINGHAM, K., CANAFAX, D.M., MATAS, A.J., DUNN, D.L. (1991). Cancer development in renal allograft recipients treated with conventional and cyclosporin immunosuppression. *Transplantation Proc.*, **73**, 1104-1105
- GUSTERSON, B.A., ANBAZHAGAN, R., WARREN, W., MIDGELY, C., LANE, D.P., O'HARE, M., STAMPS, A., CARTER, R. & JAYATILAKE, H. (1991). Expression of p53 in premalignant and malignant squamous epithelium. *Oncogene*, **6**, 1785-1789
- HALEVY, O. (1993). p53 gene is up-regulated during skeletal muscle cell differentiation. *Biochemical and Biophysical Research Communications*, **192**, 714-719
- HALL, P.A., McKEE, P.H., DU P-MENAGE, H., DOVER, R. & LANE, D.P. (1993). High levels of p53 protein in UV-irradiated normal human skin. *Oncogene*, **8**, 203-207
- HALL, P.A., LANE, D.P. (1994). p53 in tumour pathology - can we trust immunocytochemistry? - revisited. *J. Pathol.*, **172**, 1-4
- HALPERT, R., FRUCHTER, R.G., SEDLIS, A., BUTT, K., BOYCE, J.G., SILLMAN, F.H. (1986). Human papillomavirus and lower genital neoplasia in renal transplant patients. *Obstet. Gynecol.*, **68**, 251-258



- HANAHAN, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.*, **166**, 557-580
- HARDIE, I.R., STRONG, R.W., HARTLEY, L.C.J., WOODRUUFF, P.W.H., CLUNIE, G.J.A. (1980). Skin cancer in Caucasian renal allograft recipients living in a subtropical climate. *Surgery*, **87**, 177-183
- HARPER, J.R., REYNOLDS, S.H., GREENHALGH, D.A., STRICKLAND, J.E., LACAL, J.C., YUSPA, S.H. (1987). Analysis of the ras Ha oncogene and its p21 product in chemically induced skin tumours and tumour derived cell lines. *Carcinogenesis*, **8**, 1821-1825
- HARPER, J.W., ADAMI, G.R., WEI, N., KEYOMARSI, K., ELLEDGE, S.J. (1993). The p21 cdk-interacting protein CIP 1 is a potent inhibitor of G1 cyclin dependent kinases. *Cell*, **75**, 805-816
- HARRIS, A.L. (1991). Telling changes of base. *Nature*, **350**, 377-378
- HARTEVELT, M.M., BOUWES BAVINCK, J.N., KOOTTE, A.M.M., VERMEER, B.J., VANDENBROUCKE, J.P. (1990). Incidence of skin cancer after renal transplantation in the Netherlands. *Transplantation*, **49**, 506-509
- HARTLEY, J.A., GIBSON, N.W., ZWELLING, L.A., YUSPA, S.H. (1985). Association of DNA strand breaks with accelerated terminal differentiation in mouse epidermal cells exposed to tumour promoters. *Cancer Res.*, **45**, 4864-4870
- HARTLEY, J.A., GIBSON, N.W., KILKENNY, A., YUSPA, S.H. (1987). Mouse keratinocytes derived from initiated skin or papillomas are resistant to DNA strand breakage by benzoyl peroxide: a possible mechanism for tumour promotion by benzoyl peroxide. *Carcinogenesis*, **8**, 1827-1830
- HARTWELL, L. (1992). Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell*, **71**, 543-546
- HARVEY, M., McARTHUR, M.J., MONTGOMERY, C.A., BUTEL, J.S., BRADELY, A., DONEHOWER, L.A. (1993). Spontaneous and carcinogen-induced tumourigenesis in p53 deficient mice. *Nature Genetics*, **5**, 225-229
- HASHIDA, T., YASUMOTO, S. (1991). Induction of chromosome abnormalities in mouse and human epidermal keratinocytes by the human papillomavirus type 16 E7 oncogene. *J. Gen. Virol.*, **72**, 1569-1577
- HAUGEN, T.H., CRIPE, T.P., GINDER, G.D., KARIN, M., TUREK, L.P. (1987). Trans-activation of an upstream early gene promoter of bovine papillomavirus-1 by a product of the viral E2 gene. *EMBO. J.*, **6**, 145-152

- HAWLEY-NELSON, P., ANDROPHY, E.J., LOWY, D.R., SCHILLER, J.T. (1988). The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2 dependent enhancer. *EMBO. J.*, **7**, 525-531
- HAWLEY-NELSON, P., VOUSDEN, K.H., HUBBERT, N.L., LOWRY, D.R., SCHILLER, J.T. (1989). HPV E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO. J.*, **8**, 3905
- HAYASHI, K. (1992). PCR-SSCP: a method for detection of mutations. *GATA*, **9**, 73-79
- HECK, D.V., YEE, C.L., HOWLEY, P.M., MUNKER, K. (1992). Efficiency of binding the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomaviruses. *Proc. Natl. Acad. Sci. USA*, **89**, 4442-4446
- HEILMAN, L.A., LAW, M.F., ISRAEL, M.A., HOWLEY, P.M. (1980). Cloning of human papilloma virus genomic DNAs and analysis of homologous polynucleotide sequences. *J. Virol.*, **36**, 395-407
- HELANDER, S.D., PETERS, M.S., PITTELKOW, M.R. (1993). Expression of p53 protein in benign and malignant epidermal pathologic conditions. *J. Am. Acad. Dermatol.*, **29**, 741-748
- HENNINGS, H., BEN, T., YUSPA, S.H. (1984). Treatment of epidermal cell lines with 12-o-tetradecanoyl phorbol-13-acetate (TPA): lack of differentiative response and variable proliferative response. *Proc. Am. Assoc. Cancer Res.*, **25**, 146
- HENNINGS, H., YUSPA, S.H. (1985). 2 Stage tumour promotion in mouse skin - an alternative interpretation. *J. Natl. Cancer Inst.* **74**, 735-740
- HINDS, P., FINLAY, C., LEVINE, A.J. (1989). Mutation is required to activate the p53 gene for cooperation with the ras oncogene in transformation. *J. Virol.*, **63**, 739-746
- HIRSCH-BEHNAM, A., DELIUS, H., DE VILLIERS, E.M. (1990). A comparative sequence analysis of two human papillomavirus (HPV) types 2a and 57. *Virus Res.*, **18**, 81-98
- HJORTH, R., MORENO-LOPEZ, J. (1982). Purification of bovine papillomavirus by gel filtration on sephacryl S-1000 superfine. *J. Virol. Methods*, **5**, 151-158
- HOLGATE, C.S., JACKSON, P., POLLARD, K., LUNNY, D., BIRD, C.C. (1986). Effect fixation on T and B lymphocyte surface membrane antigen demonstration in paraffin processed tissue. *J. Pathol.*, **149**, 293-300.

- HOLLSTEIN, M.C., METCALF, R.A., WELSH, J.A., MONTESANO, R., HARRIS, C.C. (1990). Frequent mutation of the p53 gene in human esophageal cancer. *Proc. Natl. Acad. Sci. USA*, **87**, 9958-9961.
- HOLLSTEIN, M., SIDRANSKY, D., VOGELSTEIN, B., HARRIS, C.C. (1991). P53 mutations in human cancers. *Science*, **253**, 49-53
- HORWITZ, B.H., WEINSTAT, D.L., DIMAIO, D. (1989). Transforming activity of a 16-amino-acid segment of the bovine papillomavirus E5 protein linked to a random sequence of hydrophobic amino acids. *J. Virol.*, **63**, 4515-4519
- HOWLEY, P.M. (1991). Role of human papillomaviruses in human cancer. *Cancer Res. (suppl)*, **51**, 5019s-5022s
- HOXTELL, E.O., MANDEL, J.S., MURRAY, S.S., SCHUMAN, L.M., GOLTZ, R.W. (1977). Incidence of skin carcinoma after renal transplantation. *Arch. Dermatol.*, **113**, 436-438
- HUBBERT, N.L., SCHILLER, J.T., LOWY, D.R., ANDROPHY, E.J. (1988). Bovine papilloma virus-transformed cells contain multiple E2 proteins. *Proc. Natl. Acad. Sci. USA*, **85**, 5864-5868
- HUITBREGTSE, J.M., SCHEFFNER, M., HOWELY, P.M. (1991). A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO. J.*, **10**, 4129-4135
- HURLIN, P.J., KAURZ, P., SMITH, P.P., PEREZ-REYES, N., BLANTEN, R.R., McDOUGALL, J.K. (1991). Progression of human papillomavirus type 18-immortalized human keratinocytes to a malignant phenotype. *Proc. Natl. Acad. Sci. USA*, **88**, 570-574
- IFTNER, T., BIERFELDER, S., CSAPO, Z., PFISTER, H. (1988). Involvement of HPV type 8 genes E6 and E7 in transformation and replication. *J. Virol.*, **62**, 3655-3661.
- IFTNER, T., SAGNER, G., PFISTER, H., WETTISTER, F.O. (1990). The E7 protein of human papillomavirus 8 is a nonphosphorylated protein of 17KDA and can be generated by two different mechanisms. *Virology*, **174**, 428-436.
- IGGO, R., GATTER, K., BARTEK, J., LANE, D.P., HARRIS, A.L. (1990). Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet*, **335**, 675-679
- JABLONSKA, S., DABROWSKI, J., JAKUBOWICZ, K. (1972). Epidermodysplasia verruciformis as a model in studies on the role of papova viruses in oncogenesis. *Cancer Res.*, **32**, 583-589

- JARRETT, W.F., CAMPO, M.S., O' NEIL, B.W., LAIRD, N.M., MOAR, M.H., SARTIRANA, M.I. (1984). Alimentary fibropapilloma in cattle. A spontaneous tumour, nonpermissive for papillomavirus replication. *J. Natl. Cancer Inst.*, **73**, 499-504
- JENKINS, J.R., RUDGE, K., CURRIE, G.A. (1984). Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. *Nature*, **312**, 651-654
- JERRY, D.J., OZBURN, M.A., KITTRELL, F.S., LANE, D.P., MEDINA, D., BUTEL, J.S. (1993). Mutations in p53 are frequent in the pre-neoplastic stage of mouse mammary tumour development. *Cancer Res.*, **53**, 3374-3381
- JOHNSON, T.M., ROWE, D.E., NELSON, B.R., SWANSON, N.A. (1992). Squamous cell carcinoma of the skin (excluding lip and oral mucosa). *J. Am. Acad. Dermatol.*, **26**, 467-484
- JONES, E.H., BIGGAR, R.J., NKURUMAH, F.K., LAWLER, S.D. (1980). Study of the HLA system in Burkitts Lymphoma. *Hum. Immunol.*, **3**, 207-210
- JUVEN, T., BARAK, Y., ZAUBERMAN, A., GEORGE, D.L., MOREN, J.L. (1993). Wild type p53 can mediate sequence-specific transactivation of an internal promoter within the MDM2 gene. *Oncogene*, **8**, 3411-3416
- KASTAN, M.B., ONYEKWERE, O., SIDRANSKY, D., VOGELSTEIN, B. & CRAIG, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, **51**, 6304-6311
- KASTAN, M.B., ZHAN, A., EI-DEIRY, W.S., CARRIER, F., JACHS, T., WALSH, W.V., PLUNKETT, B.S., VOGELSTEIN, B., FORNACER, A.J. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, **71**, 587-597
- KAUFFMANN, W.K., KAUFFMANN, D.G. (1993). Cell Cycle control, DNA repair and initiation of carcinogenesis. *FASEB. J.*, **7**, 1188-1191
- KEMP, C.J., DONEHOWER, L.A., BRADLEY, A., BALMAIN, A. (1993). Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically - induced skin tumours. *Cell*, **74**, 813-822
- KERN, S.E., FINZLER, K.W., BRUSKIN, A., JAROSZ, D., FRIEDMAN, P., PRIVES, C., VOGELSTEIN, B. (1991). Identification of p53 as a sequence - specific DNA - binding protein. *Science*, **252**, 1708-1711
- KERN, S.S.E., PIETENPOL, J.A., THIAGALINGAM, S., SEYMOUR, A., KINZLER, K.W., VOGELSTEIN, B. (1992). Oncogenic forms of p53 inhibit p53 regulated gene expression. *Science*, **256**, 827-830



- KESSIS, T.D., SLEBOS, R.J., NELSON, W.G., KASTAN, M.B., PLUNKETT, B.S., HAN, S.M., LORINCZ, A.T., HEDRICK, L., CHO, K.R. (1993). Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. *Proc. Natl. Acad. Sci. USA.*, **90**, 3988-3992
- KHANNA, K.K., LAVIN, M.F. (1993). Ionizing radiation and UV induction of p53 protein by different pathways in ataxia-telangiectasia cells. *Oncogene*, **8**, 3307-3312
- KHOCHBIN, S., PRINCIPAUD, E., CHABANAS, A., LAWRENCE, J.J. (1988). Early events in murine erythroleukemia cells induced to differentiate - accumulation and gene-expression of the transformation-associated cellular protein p53. *J. Mol. Biol.*, **200**, 55-64
- KHOCHBIN, S., LAWRENCE, J.J. (1989). An RNA involved in p53 messenger RNA maturation in murine erythroleukemia cells induced to differentiate. *EMBO. J.*, **8**, 4107-4114
- KIYONO, T., HIRAIWA, A., ISHIBASHI, M. (1992). Differences in transforming activity and coded amino acid sequence among E6 genes of several papillomaviruses associated with epidermodysplasia verruciformis. *Virology*, **186**, 628-639
- KNUDSON, A.G. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl. Acad. Sci. USA.*, **68**, 820-823
- KOMLY, K.A., BREITBURD, F., CROISSANT, O., STREECK, R.E. (1986). The L1 open reading frame of human papillomavirus type-1A encodes a minor structural protein carrying type specific antigens. *J. Virol.*, **60**, 813-816
- KRAEMER, K.H., LEE, M.M., SCOTTO, J. (1984). DNA repair protects against internal and cutaneous neoplasia-evidence from xeroderma pigmentosum. *Carcinogenesis*, **5**, 511-514
- KRAISS, S., QUAISRE, A., OREN, M., MONTENARH, M. (1988). Oligomerisation of oncoprotein-p53. *J. Virol.*, **62**, 4737-4744
- KRAJINOVIC, M., LAZIC, J., STANIMIROVIC, B., DIKLIC, V., SAVIC, A.. (1993). The E2 region of HPV 16 in relation to different types of cervical lesions. *J. Med. Virol.*, **41**, 1-5
- KREIDER, J.W., HOWETT, M.K., LEUREDUPREE, A.E., ZAINO, R.J., WEBER, J.A. (1987). Laboratory production in vivo of infectious human papillomavirus type-11. *J. Virol.*, **61**, 590-593



- KREMSDORF, D., JABLONSKA, S., FAVRE, M., ORTH, G. (1983). Human papillomaviruses associated with epidermodysplasia verruciformis. II. Molecular cloning and biochemical characterization of human papillomavirus 3a, 8, 10 and 12 genomes. *J. Virol*, **48**, 340-351
- KREMSDORF, D., FAVRE, M., JABLONSKA, S., OBALEK, S., RUEDA, L.A., LUTZNER, M.A., BLANCHET-BARDON, C., VADER, P.C.V.V., ORTH, G. (1984). Molecular cloning and characterization of the genomes of nine newly recognized human papillomavirus types associated with epidermodysplasia verruciformis. *J. Virol*, **52**, 1013-1018
- KRIPKE, M.L. (1984). Immunological unresponsiveness induced by ultraviolet radiation. *Immunological Rev*, **80**, 87-103
- KRUBKE, J., KRAUS, J., DELIUS, H., CHOW, L., BROCKER, T., IFTNER, T., PFISTER, H. (1987). Genetic relationship among human papillomaviruses associated with benign and malignant tumours of patients with epidermodysplasia verruciformis. *J. Gen. Virol.*, **68**, 3091-3103
- KUERBITZ, S.J., PLUNKETT, B.S., WALSH, M.V., KASTAN, M.B. (1992). Wild type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA.*, **89**, 7491-7495
- KULESZ-MARTIN, M., KILKENNY, A.E., HOLBROOK, K.A., DIGEMES, V., YUSPA, S.H. (1983). Properties of carcinogen altered mouse epidermal cells resistant to calcium-induced terminal differentiation. *Carcinogenesis*, **4**, 1367-1377
- KWA, R.E., CAMPANA, K., MOY, R.L. (1992). Biology of cutaneous squamous cell carcinoma. *J. Am. Acad. Dermatol.*, **26**, 1-26
- LAMBERT, P.F., SPANHOLZ, B.A. & HOWELY, P.M. (1987). A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. *Cell*, **50**, 69-78
- LAMBERT, P.F., DOSTATNI, N., MCBRIDE, A., YANIV, M., HOWLEY, P.M. (1989a). Functional analysis of the papillomavirus E2 trans-activator in *Saccharomyces cerevisiae*. *Genes Dev.*, **3**, 38-48
- LAMBERT, P.F., HUBBERT, N.L., HOWLEY, P.M., SCHILLER, J.T. (1989b). Genetic assignment of multiple E2 gene products in bovine papillomavirus-transformed cells. *J. Virol.*, **63**, 3151-3154
- LAMBERT, P.F. (1991). Papillomavirus DNA replication. *J. Virol.*, **65**, 3417-3420
- LANCASTER, D., OLSON, C. (1982). Animal papillomaviruses. *Microbiol. Rev.*, **46**, 191-207

- LANE, D.P. (1992). p53, guardian of the genome. *Nature*, **358**, 15-16
- LANE, D.P. (1993). A death in the life of p53. *Nature*, **362**, 786-787
- LANE, D.P., CRAWFORD, L.V. (1979). T antigen is bound to a host protein in SV40 transformed cells. *Nature*, **278**, 261-263
- LAVIGUER, A., MAITBY, V., MOCH, D., ROSSANT, J., DAWSON, T., BERNSTEIN, A. (1989). High incidence of lung, bone and lymphoid tumours, in transgenic mice overexpressing mutant alleles of the P53 oncogene. *Mol. Cell. Biol.*, **9**, 3982-3991.
- LAZO, P.A., GALLEG0, M.I., BALLESTER, S., FEDUCHI, E. (1992). Genetic alterations by human papillomaviruses in oncogenesis. *FEBS*, **300**, 109-113
- LECHNER, M.S., MACK, D.H., FINICLE, A.B., CROOK, T., VOUSDEN, K.H., LAIMINS, A. (1992). Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53-mediated repression of transcription. *EMBO J.*, **11**, 3045-3052
- LEDO, E. (1992). Warts, keratoacanthoma and squamous cell carcinoma: A spectrum of the same neoplastic process ? *Int. J. Dermatol.*, **31**, 777-778
- LEECHANACHAI, P., BANKS, L., MOREAU, F., MATLASHEWSKI, G. (1992). The E5 gene from human papillomavirus type 16 is an oncogene which enhances growth factor-mediated signal transduction to the nucleus. *Oncogene*, **7**, 19-25
- LENNARD, L., THOMAS, M.J., HARRINGTON, C.I., MADDOCKS, J.L. (1985). Skin cancer in renal transplant patients is associated with increased concentrations of 6-thioguanine nucleotide in red blood cells. *Br. J. Dermatol.*, **113**, 723-729
- LEVINE, A.J., MOMAND, J. (1990). Tumour suppressor genes: The P53 and retinoblastoma sensitivity genes and gene products. *Biochimica et Biophysica acta.*, **1032**, 119-136
- LEVINE, A.J., MOMAND, J., FINDLAY, C.A. (1991). The P53 tumour suppressor gene. *Nature*, **351**, 453-456.
- LEVINE, A.J., (1993), The P53 tumour suppressor gene and product. 11 Ernst Klenk lecture. *Biol. Chem. Hoppe-Seyler*, **374**, 227-235.
- LI, F.P., FRAUMENI, J.F. (1969). Soft-tissue sarcomas, breast cancer and other neoplasms. A familial syndrome? *Ann. Intern. Med.*, **71**, 747-752
- LINDER, S., MARSHALL, H. (1990). Immortalisation of primary cells by DNA tumour viruses. *Exp. Cell Res.*, **191**, 1-7

- LINZER, D.H.I., LEVINE, A.J. (1979). Characterisation of a 54 Kdalton cellular SV40 tumour antigen present in SV40 transformed cells and uninfected embryonal carcinoma cells. *Cell*, **17**, 43-52
- LIU, X., MILLER, C.W., KOEFFLER, P.H., BERK, A.J. (1993). The p53 activation domain binds the TATA box-binding polypeptide in Hollo - TFIID, and a neighboring p53 domain inhibits transcription. *Mol. Cell. Biol.*, **13**, 3291-3300
- LORINCZ, A.T., REID, R., JENSON, A.B., GREENBERG, M.D., LANCASTER, W.D., KURMAN, R.J. (1992). Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. *Obstet. Gynecol.* **79**, 328-337
- LOWE, S.W., SCHITT, E.M., SMITH, S.W., OSBORNE, B.A., JACHS, T. (1993a). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362**, 847-849
- LOWE, S.W., RULEY, H.E., JACHS, T., HOUSMAN, D.E. (1993b). p53-dependent apoptosis modulates the cytotoxicity of anti cancer agents. *Cell*, **74**, 957-967
- LOWE, S.W., RULEY, H.E. (1993). Stabilization of the p53 tumour suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes and Dev.*, **7**, 535-545
- LU, X., PARK, S.H., THOMPSON, T.C., LANE, D.P. (1992). ras-induced hyperplasia occurs with mutation of p53 but an activated ras and myc together can induce carcinoma without p53 gene mutation. *Cell*, **70**, 153-161
- LUDLOW, J.W. (1993). Interaction between SV40 large-tumour antigen and the growth suppressor protein pRB and protein p53. *FASEB. J.*, **7**, 865-871
- LUNGU, O., WRIGHT, T.C., SILVERSTEIN, S. (1992). Typing of human papillomavirus by polymerase chain-reaction amplification with L1 consensus primers and RFLP ananlysis. *Molecular Cellular Probes*, **2**, 145-152
- LUTZNER, M., CROISSANT, O., DUCASSE, M.F., KREIS, H., CROSNIER, J., ORTH, G. (1980). A potentially oncogenic human papillomavirus (HPV-5) found in two renal allograft recipients. *J. Invest. Dermatol.*, **75**, 353-356
- LUTZNER, M.A., ORTH, G., DUTRONQUAY, V. (1983). Detection of human papillomavirus type 5 DNA in skin cancers of an immunosuppressed renal allograft recipient. *Lancet*, **I**: 422-424
- LYNCH, P.J. (1982). Warts and cancer. The oncogenic potential of human papillomaviruses. *Am. J. Dermatopathol.*, **4**, 55-60
- MACKAY, J., STEEL, C.M., ELDER, P.A., FORREST, A.P.M., EVANS, H.J. (1988). Allele loss on short arm of chromosome 17 in breast cancers. *Lancet*, 1384-1385

- MALKIN, D., LI, F.P., STRONG, L.C., FRAUMENI, J.F., NELSON, C.E., KIM, D.H., KASSEL, J., GRYKA, M.A., BISCHOFF, F.Z., TAINSKY, M.A., FRIEND, S.H. (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science*, **250**, 1233-1238
- MANOS, M., TING, M.Y., WRIGHT, D.K., LEWIS, A.J., BROKER, T.R., WOLINSKY, S.M. (1989). The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells*, **7**, 209-214
- MARKS, R., FOLEY, P., GOODMAN, G., HAGE, B.H. & SELWOOD, T.S. (1986). Spontaneous remission of solar keratoses - the case for conservative management. *Br. J. Dermatol.*, **115**, 649-655
- MARKS, R., RONNIE, G., SELWOOD, T. (1988). The relationship of basal cell carcinomas and squamous cell carcinomas to solar keratoses. *Arch. Dermatol.*, **124**, 1039-1042
- MARKS, R. (1992). Sun damaged skin. Published by Martin Dunity.
- MASHIYAMA, S., SEHIYA, T., HAYASHI, K. (1990). Screening of multiple DNA samples for detection of sequence changes. *Technique*, **2**, 304-306
- MATLASHEWSKI, G., SCHNEIDER, J., BANKS, L., JONES, N., MURRAY, A., CRAWFORD, L. (1987). Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. *EMBO. J.*, **6**, 1741-1746
- MATLASHEWSKI, X.G. (1989). The cell biology of human papillomavirus transformed cells. *Anticancer Res.*, **9**, 1447-1556
- MCBRIDE, A.A., SCHLEGEL, R., HOWLEY, P.M. (1988). The carboxy-terminal domain shared by the bovine papillomavirus E2 transactivator and repressor contains a specific DNA binding activity. *EMBO. J.*, **7**, 533-539
- MCBRIDE, A.A., ROMANCZUK, H., HOWLEY, P. (1991). The papillomavirus E2 regulatory proteins. *J. Biol. Chem.*, **266**, 18411-18414
- McGREGOR, J.M., YU, C.C-W., DUBLIN, E.A., LEVISON, D.A., MacDONALD, D.M. (1992). Aberrant expression of p53 tumour-suppressor gene in non-melanoma skin cancer. *Br. J. Dermatol.*, **127**, 463-469
- McGREGOR, W.G., CHEN, R.H., LURKASH, L., MAHER, V.M., McCORMICK, J.J. (1991). Cell cycle dependant strand bias for UV-induced mutations in the transcribed strand of excision repair - proficient human fibroblasts, but not in repair-deficient cells. *Mol. Cell Biol.*, **11**, 1927-1934



- McLELLAND, J., REES, A., WILLIAMS, G., CHU, T. (1988). The incidence of immunosuppression related skin disease in long-term transplant patients. *Transplantation*, **46**, 871-874
- MEISELS, A., FORTIN, R. (1976). Condylomatous lesions of the cervix and vagina I. Cytologic patterns. *Acta. Cytol.*, **20**, 505-509
- MERCER, W.E., SHEILDS, M.J., AMIN, M., SAUVE, G.J., APPELLA, E., ROMANO, J.W., ULLRICH, S.J. (1990). Negative growth regulation in a glioblastoma tumour cell line that conditionally expresses human wild type p53. *Proc. Natl. Acad. Sci. USA.*, **87**, 6166-6170
- MEYERS, C., FRATTINI, M.G., HUDSON, J.B., LAIMINS, L.A. (1992). Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. *Science*, **257**, 971-973
- MIDGLEY, C.A., FISHER, C.J., BARTEK, J., VOJTESEK, B., LANE, D., BARNES, D.M. (1991). Analysis of p53 expression in human tumours. An antibody raised against human p53 expressed in Escherichia-coli. *J. Cell Science*, **101**, 183-187
- MILBURN, P.B., BRANDSMA, J.L., GLODSMAN, C.I., TEPLITZ, E.D., HEILMANN, E.J. (1988). Disseminated warts and evolving squamous cell carcinoma in a patient with aquired immunodeficiency syndrome. *J. Am. Acad. Dermatol.*, **19**, 401-405
- MILNER, J., COOK, A. (1986). Visualisation, by immunocytochemistry, of p53 at the plasma membrane of both non-transformed and SV40-transformed cells. *EMBO. J.*, **9**, 2885-2889
- MILNER, J., MEDCALF, E.A. (1991). Cotranslation of activated mutant p53 with wild type drives the wild type p53 protein into a mutant conformation. *Cell*, **65**, 774-785
- MILNER, J., MEDCALF, E.A., COOK, A.C. (1991). Tumour suppressor p53: analysis of wild type and mutant p53 complexes. *Mol. Cell. Biol.*, **11**, 12-19
- MITRANIROSENBAUM, S., TSVIELI, R. (1994). Mutations at the p53 gene occur at the latest stages of cervical malignant progression. *J. Cell. Biochem.*, **S18C**, 225
- MOLES, J.P., MOYRET, C., GUILLOT, B., JEANTEUR, P., THEILLET, C., BASSET-SEGUIN, N. (1993). p53 gene mutation in human epithelial skin cancers. *Oncogene*, **8**, 583-588
- MOMAND, J., ZAMBETTI, G.P., OLSON, D.C., GEORGES, D.L. & LEVINE, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53 mediated transactivation. *Cell*, **69**, 1237-1245



- MONINI, P., BLITZ, I.L., CASSAI, E. (1993). Cooperative DNA binding of the BPV E2 transcriptional activator is antagonized by truncated E2 polypeptides. *J. Virol.*, **67**, 5668-5676
- MONTENARH, M., KOHLER, M., HENNING, R. (1986). Complex formation of Simian virus-40 large T-antigen with cellular protein-p53. *J. Virol.*, **60**, 761-764
- MONTENARH, M. (1992). Biochemical properties of the growth suppressor/oncoprotein p53. *Oncogene*, **7**, 1673-1680
- MORAN, E. (1993). Interaction of adenoviral proteins with pRB and p53. *FASEB*, **7**, 880-885
- MOWAT, M., CHENG, A., KIMURA, N., BERNSTEIN, A., BENCHIMOL, S. (1985). Rearrangements of the cellular p53 gene in erythroleukaemic cells transformed by Freund-virus. *Nature*, **314**, 633-636
- MUKHTAR, H., BICKERS, D.R. (1993). Environmental skin cancer: mechanisms, models and human relevance. *Cancer Res*, **53**, 3439-3442
- MUNGER, K., PHELPS, W.C., BUBB, V., HOWLEY, P.M. (1989). The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J. Virol.*, **63**, 4417-4421
- MUNGER, K., PHELPS, W.C. (1993). The human papillomavirus E7 protein as a transforming and transactivating factor. *Biochimica et Biophysica acta.*, **1155**, 111-123
- NAGANO, T., VEDA, M., ICHIHASHI, M. (1993). Expression of p53 protein is an early event in ultraviolet light-induced cutaneous squamous cell carcinogenesis. *Arch. Dermatol.*, **129**, 1157-1161
- NEWTON, J.A., CAMPLEJOHN, R.S., GIBBON, D.H. (1987). A flow cytometric study of the significance of DNA aneuploidy in cutaneous lesions. *Br. J. Dermatol.*, **116**, 171-174
- NIGRO, J.M., BAKER, S.J., PREISINGER, A.C., JESSUP, J.M., HOSTETTER, R., CLEARY, K., BIGNER, S.H., DAVIDSON, N., BAYLIN, S., DEVILEE, P., GLOVER, T., COLLINS, F.S., WESTON, A., MODALI, R., HARRIS, C.C., VOGELSTEIN B. (1989). Mutations in the p53 gene occur in diverse human tumour types. *Nature*, **342**, 705-708
- NOONAN, F.P., DE FABO, E.C. (1992). Immunosuppression by ultraviolet B radiation: Initiation by urocanic acid. *Immunol. Today*, **13**, 250-254

- NOWELL, P.C. (1976). The clonal evolution of tumour cell populations. *Science*, **194**, 23-28
- OLINER, J.D., KINZLER, K.W., MEITZER, P.S., GEORGES, D.L., VOGELSTEIN, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature*, **358**, 80-83
- OLINER, J.D., PIETENPOL, J.A., THIAGALINGAM, S., GYURIS, J., KINZLER, K.W., VOGELSTEIN, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature*, **362**, 857-860
- OLSON, D.C., MARECHAL, V., MOMAND, J., CHEN, J., ROMOCKI, C., LEVINE, A.J. (1993). Identification and characterisation of multiple mdm-2 proteins and mdm-2-p53 protein complexes. *Oncogene*, **8**, 2353-2360
- OREN, M., MALTZMAN, W.E., LEVINE, A.J. (1981). Post-translation regulation of the 54K cellular antigen in normal and transformed cells. *Mol. Cell Biol.*, **1**, 101-110
- ORITA, M.Y., SUZUKI, Y., SEKIYA, T., HAYASHI, K. (1989). Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics*, **5**, 874-879
- ORTH, G., JABLONSKA, S., JARZABEK-CHORZELSKA, M., OBALEK, S., RZESA, G., FAVRE, M., CROISSANT, O. (1979). Characteristics of the lesions and risk of malignant conversion associated with the type of human papillomavirus involved in epidermodysplasia verruciformis. *Cancer Res.* **39**, 1074-1082
- ORTH, G. (1986). Epidermodysplasia verruciformis. in *The papoviridae*, vol 2. *The papillomaviruses* (Salzman, N.P and Howley, P.M, editors). Plenum: New York
- OSTROW, R.S., BENDER, M., NIMURA, M., SEKI, T., KAWASHIMA, M., PASS, F., FARAS, A.J. (1982). Human papillomavirus DNA in cutaneous primary and metastasized squamous cell carcinomas from patients with epidermodysplasia verruciformis. *Proc. Natl. Acad. Sci. USA*, **79**, 1634-1638
- OSTROW, R., ZACHOW, K., WATTS, S., BENDER, M., PASS, F., FARAS, A. (1983). Characterization of two HPV 3 related papillomaviruses from common warts that are distinct from flat warts or epidermodysplasia verruciformis. *J. Invest. Dermatol.*, **80**, 436-440
- OSTROW, R., MANIAS, D., MITCHELL, A. (1987). Epidermodysplasia verruciformis: A case associated with primary lymphatic dysplasia, depressed cell-mediated immunity, and Bowens disease containing human papillomavirus 16 DNA. *Arch. Dermatol.*, **123**, 1511-1516

- OSTROW, R.S., SHAVER, K., TURNQUIST, S., VIKSNINS, A., BENDER, M., VANCE, C., KAYE, V., FARAS, A.J. (1989). Human papillomavirus-16 DNA in a cutaneous invasive cancer. *Arch. Dermatol.*, **125**, 666-669
- PALEFSKY, J.M., GONZALES, J., GREENBLATT, R.M., AHN, D.K., HOLLANDER, H. (1990). Anal intraepithelial neoplasia and anal papillomavirus infection among homosexual males with group-IV HIV disease. *J. Am. Med. Assoc.* **263**, 2911-2916
- PARADA, L.F., LAND, H., WEINBERG, R.A., WOLF, D. ROTTER, V. (1984). Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. *Nature*, **312**, 649-651
- PARTON, A., GRAND, R.J.A., BIGGS, P., SETTLEMAN, J., DIMAIO, D., GALLIMORE, P.H. (1990). Integrated HPV 1 genomes in a human keratinocyte cell line can be transactivated by a SV40/BPV1 recombinant virus which expresses BPV1 E2 proteins. *Virology*, **175**, 508-517
- PATER, M.M., DUNNE, J., HOGAN, G., GHATAGE, P., PATER, A. (1986). Human papillomaviruses type 16 and 18 sequences in early cervical neoplasia. *Virology*, **155**, 13-18
- PATER, M.M., NAKSHATI, H., KISAKA, C., PATER, A. (1992). The first 124 nucleotides of the E7 coding sequence of HPV 16 can render the HPV 11 genome transformation competent. *Virology*, **186**, 348-351
- PENN, I. (1991). Cancer in the immunosuppressed organ recipient. *Transplantation Proc.*, **23**, 1771-1772
- PENNICA, D., GOEDDEL, D.V., HAYFLICKS, J.S., REICH, N.C., ANDERSON, C.W., LEVINE, A.J. (1984). The amino acid sequence of murine p53 determined from a cDNA clone. *Virology*, **134**, 477-482
- PENNIE, W.D., CAMPO, M.S. (1992). Synergism between bovine papillomavirus type 4 and the flavenoid quercetin in cell transformation in vitro. *Virology*, **190**, 861-865
- PENNIE, W.D., GRINDLAY, G.J., CAIRNEY, M., CAMPO, M.S. (1993). Analysis of the transforming functions of bovine papillomavirus type 4. *Virology*, **193**, 614-620
- PERRY, M.E., LEVINE, A.J. (1993). Tumor-suppressor P53 and the cell cycle. *Current Opinion in Genetics and Development*. **3**, 50-54.
- PFISTER, H., NURNBERGER, F., GISSMANN, L., ZUR HAUSEN, H. (1981). Characterisation of a human papillomavirus from epidermodysplasia verruciformis lesions of a patient from Upper Volta. *Int. J. Cancer*, **27**, 645-650

- PFISTER, H., GASSENMAIER, A., NURNBERGER, F., STUTTGEN, G. (1983a). Human papilloma virus 5-DNA in a carcinoma of an epidermodysplasia verruciformis patient infected with various human papillomavirus types. *Cancer Res.* **43**, 1436-1441
- PFISTER, H., HETTICH, I., RUNNE, U., GISSMANN, L., CHILF, G.N. (1983b). Characterization of human papillomavirus type 13 from focal epithelial hyperplasia Heck lesions. *J. Virol.* **47**, 363-366
- PHELPS, W.C., YEE, C.L., MUNGER, K., HOWLEY, P.M. (1988). The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. *Cell*, **53**, 539-547
- PIERCEALL, W.E., MUKHOPADHYAY, T., GOLDBERG, L.H., ANANTHASWAMY, H.N. (1991a). Mutations in the p53 tumour suppressor gene in human cutaneous squamous cell carcinomas. *Mol. Carcinog.*, **4**, 445-449
- PIERCEALL, W.E., GOLDBERG, L.H., TAINSKY, M.A., MUKHOPADHYAY, T., ANANTHASWAMY, H.N. (1991b). Ras gene mutation and amplification in human non-melanoma skin cancer. *Mol. Carcinogenesis*, **4**, 196-202
- PIETENPOL, J.A., VOGELSTEIN, B. (1993). No room at the p53 inn. *Nature*, **365**, 17-18
- PRIVES, C., MANFREDI, J.T. (1993). The P53 tumour suppressor protein: Meeting review. *Genes and Development*, **7**, 529-534.
- PURDIE, C.A., O'GRADY, J., PIRIS, J., WYLLIE, A.H., BIRD, C.C. (1991). p53 expression in colorectal tumours. *Am. J. Pathol.*, **138**, 807-813
- PURDIE, C.A., HARRISON, D.J., PETER, A., DOBBIE, L., WHITE, S., HOWIE, S.E.M., SALTER, D.M., BIRD, C.C., WYLLIE, A.H., HOOPER, M.L., CLARKE, A.R. (1994). Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. *Oncogene*, **9**, 603-609
- QUAN, M.B., MOY, R.L. (1991). The role of human papillomaviruses in carcinoma. *J. Am. Acad. Dermatol.*, **25**, 698-705
- QUINTANILLA, M., BROWN, K., RAMSDEN, M., BALMAIN, A. (1986). Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. *Nature*, **322**, 78-80
- RANDALL, M.B., GEISINGER, K.R., KUTE, T.E., BUSS, D.H., PRICHARD, R.W. (1990). DNA content and proliferative index in cutaneous squamous-cell carcinoma and keratoacanthoma. *Am. J. Clin. Pathol.*, **93**, 259-262
- RAYCROFT, I., WU, H., LOZANO, G. (1990). Transcriptional activation by wild type but not transforming mutants of the p53 anti-oncogene. *Science*, **249**, 1049-1051



- REICH, N.C., LEVINE, A.J. (1984). Growth regulation of a cellular tumour antigen, p53, in non-transformed cells. *Nature*, **308**, 199-201
- RO, Y-S., VOJTESEK, B., COOPER, P.N., LEE, J.A., HARRISON, D., ANGUS, B., REES, J., HORNE, C.H.W. & LANE, D.P. (1992). p53 protein expression in benign and malignant squamous and melanocytic skin tumours - an immunohistochemical study. *J. Invest. Dermatol.*, **98**, 540-544
- ROBERTS, S., ASHMOLE, I., JOHNSON, G.D., KREIDER, J.W., GALLIMORE, P.H. (1993). Cutaneous and mucosal HPV proteins form intermediate filament-like structures in epithelial cells. *Virology*, **197**, 176-187
- RODRIGUES, N.R., ROWAN, A., SMITH, M.E.F., KERR, I.B., BODMER, W.F., GANNON, J.V., LANE, D.P. (1990). p53 mutations in colorectal cancer. *Proc. Natl. Acad. Sci. USA.*, **87**, 7555-7559
- ROGEL, A., POPLINER, M., WELLS, C.G., OREN, M. (1985). p53 cellular tumour antigen, analysis of mRNA levels in normal adult tissues, embryos and tumours. *Mol. Cell Biol.*, **5**, 2851-2855.
- ROMANCZUK, H., THIERRY, F., HOWLEY, P.M. (1990a). Mutational analysis of cis elements involved in E2 modulation of human papillomavirus type-16 P97 and type 18 p105 promoters. *J. Virol.*, **64**, 2849-2859
- ROMANCZUK, H., THEIRRY, F., HOWLEY, P.M. (1990b). The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV 18 regulatory region in human keratinocytes. *J. Virol.*, **64**, 2849-
- ROTTER, V., BOSS, M.A., BALTIMORE, D. (1981). Increased concentration of an apparently identical cellular protein in cell transformed by either Abelson murine leukemia virus or other transforming agents. *J. Virol.*, **38**, 336-346
- ROTTER, V., FOORD, O., NAVOT, N. (1993). In search of the functions of normal P53 protein. *Trends in Cell Biology*, **3**, 46-49.
- ROWE, D.E., CARRELL, R.J., DAY, C.L. (1992). Prognostic factors for local recurrence, metastasis and survival rates in squamous cell carcinoma of the skin, ear and lip: implications for treatment modality selection. *J. Am. Acad. Dermatol.*
- RUDLINGER, R., SMITH, I.W., BUNNEY, M.H., HUNTER, J.A.A. (1986). Human papillomavirus infections in a group of renal transplant recipients. *Br. J. Dermatol.*, **115**, 681-692



- RUDLINGER, R., GROB, R., YU, Y.X., SCHNYDER, U.W. (1989). Human papillomavirus-35-positive Bowenoid papulosis of the anogenital area and concurrent human papillomavirus-35-positive verruca with Bowenoid dysplasia of the periungual area. *Arch Dermatol.* **125**, 655-659.
- RUDLINGER, R., GROB, R. (1989). Papillomavirus infection and skin cancer in renal allograft recipients. *Lancet*, **I**, 1132-1133
- RUSTIGI, A.K., DYSON, N., BERNARDS, R. (1991). Amino-terminal domains of c-myc and N-myc proteins mediate binding to the retinoblastoma gene product. *Nature*, **352**, 541-544
- SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. (1989). *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbour Laboratory Press: Cold Spring Harbor, NY. ppE.3-E.4
- SANDLER, A.B., VAN DE POL, S.B., SPALHOLZ, B.A. (1993). Repression of bovine papillomavirus type 1 transcription by the E1 replication protein. *J. Virol.*, **67**, 5079-5087
- SANG, B.C., BARBOSA, M.S. (1992). Single amino-acid substitutions in low-risk human papillomavirus (HPV) type 6 E7 protein enhance features characteristic of the high-risk HPV E7 oncoproteins. *Proc. Natl. Acad. Sci. USA.*, **89**, 8063-8067
- SCHEFFNER, M., WERNES, B.A., HULBREGTSE, J.M., LEVINE, A.J. & HOWLEY, P.M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*, **63**, 1129-1136
- SCHEFFNER, M., MUNGER, K., BYRNE, J.C. & HOWLEY, P.M. (1991). The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc. Nat. Acad. Sci. USA*, **88**, 5523-5527
- SCHEFFNER, M., TAKAHASHI, T., HUIBREGTSE, J.M., MINNA, J.D. & HOWLEY, P.M. (1992). Interaction of the human papillomavirus type 16 E6 oncoprotein with wild-type and mutant human p53 proteins. *J. Virol.*, **66**, 5100-5105
- SCHILLER, J.T., KLEINER, E., ANDROPHY, E.J., LOWRY, D.R., PFISTER, H. (1989). Identification of bovine papillomavirus mutants with increased transforming and transcriptional activity. *J. Virol.*, **63**, 1775-1782
- SCHLEGEL, R., WADE-GLASS, M. (1987). E5 transforming polypeptide of bovine papillomavirus. *Cancer Cells*, **5**, 87-91
- SCHLICHTHOLZ, B., LEGROS, Y., GILLET, D., GUILLARD, C., MARTY, M., LANE, D., CALVO, F., SOUSSI, T. (1992). The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot. *Cancer Res.*, **52**, 6380-6384

- SCHMID, P., LORENZ, A., HAMEISTER, H., MONTENARH, M. (1991). Expression of p53 during mouse embryogenesis. *Development*, **113**, 857-865
- SCHNEIDER, A. (1993). Pathogenesis of genital HPV infection. *Genitourinary Medicine*, **69**, 165-173
- SCORZA, S.R., FABIO, G., LAZZARIN, A., EISERA, N.B., MORONI, M., ZANUSSI, C. (1986). HLA associated susceptibility to acquired immunodeficiency syndrome in Italian patients with human-immunodeficiency-virus infection. *Lancet*, **II**, 1187-1189
- SCRIBNER, J.D., SCRIBNER, N.K., MCKNIGHT, B., MOTTET, N.K. (1983). Evidence for a new model of tumour progression from carcinogenesis and promotion studies with 7-bromomethylbenz(A)anthracene. *Cancer Res.*, **43**, 2034-2042
- SEDMAN, S.A., BARBOSA, M.S., VASS, W.C., HUBBERT, N.L., HAA, J.A., LOWY, D.R., SCHILLER, J.T. (1991). The full length E6 protein of HPV type 16 has transforming and trans-activating activities and cooperates with E7 to immortalise keratinocytes in culture. *J. Virol.*, **65**, 4860-4866
- SEEDORF, K., KRAMMER, G., DURST, S., SUHAI, S., ROWEKAMP, W.G. (1987). Identification of early proteins of the human papilloma viruses type 16 (HPV 16) and type 18 (HPV 18) in cervical carcinoma cells. *EMBO. J.*, **6**, 139-144
- SHAULSKY, G., BEN-ZE'EV, A., ROTTER, V. (1990). Sub-cellular distribution of the p53 protein during the cell cycle of Bulb/c 3T3 cells. *Oncogene*, **5**, 1707-1711
- SHAULSKY, S., GOLDFINGER, N., PELED, A., ROTTER, V. (1991a). Involvement of wild type p53 in pre-B cell differentiation in vitro. *Proc. Natl. Acad. Sci. USA.*, **88**, 8982-8986
- SHAULSKY, G., GOLDFINGER, N., ROTTER, V. (1991b). Alterations in tumour-development in vivo mediated by expression of wild type or mutant p53 proteins. *Cancer Res.*, **51**, 5232-5237
- SHAW, P., BOVEY, R., TARDY, S., SAHLI, R., SARDAT, B., COSTA, J. (1992). Induction of apoptosis by wild type p53 in a human colon tumour-derived cell line. *Proc. Natl. Acad. Sci. USA.*, **89**, 4495-4499
- SHEFFIELD, V.C., BECK, J.S., KWITEK, A.E., SANDSTROM, D.W., STONE, E.M. (1993). The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics*, **16**, 325-332
- SHEIL, A.G.R., FLAVEL, S., DISNEY, A.P.S., MATHEW, T.H. (1985). Cancer development in patients progressing to dialysis and renal transplantation. *Transplantation Proc.* **17**, 1685-1692

- SHEIL, A.G.R., DISNEY, A.P.S., MATHEW, T.H., AMISS, N. and EXELL, L. (1991). Cancer development in cadaveric donor renal allograft recipients treated with Azathioprine (AZA) or Cyclosporin (CYA) or AZA/CYA. *Transplantation Proc.*, **23**, 1111-1112
- SHILO, Y., YAMAMOTO, T., YAMAGICHI, N. (1993). Identification of a DNA element that can enhance p53- mediated transactivation. *Oncogene*, **8**, 2059-2065
- SHIRASAWA, H., TOMITA, Y., KUBOTA, K., KASAI, T., SEKIYA, S., TAKAMIZAWA, H., SIMIZU, B. (1988). Transcriptional differences of the human papillomavirus type16 genome between pre-cancerous lesions and invasive carcinomas. *J. Virol.*, **62**, 1022-1027
- SHUTTLEWORTH, D., MARKS, R., GRIFFIN, P.J.A. & SALAMAN, J.R. (1987). Dysplastic epidermal change in immunosuppressed patients with renal transplants. *Q. J. Med.*, **243**, 609-616
- SHUTTLEWORTH, D., MARKS, R., GRIFFIN, P.J.A., SALAMAN, J.R. (1989). Epidermal dysplasia and cyclosporin therapy in renal transplant patients: A comparison with azathioprine. *Br. J. Dermatol.*, **120**, 551-554
- SIDRANSKY, D., VON ESCHENBACH, A., TSAI, Y.C., JONES, P., SUMMERHAYES, I., MARSHALL, F., PAUL, M., GREEN, P., HAMILTON, S.R., FROST, P., VOGELSTEIN, B. (1991). Identification of p53 gene mutations in bladder cancers and urine samples. *Science*, **252**, 706-709
- SIDRANSKY, D., FROST, P., VON ESCHENBACH, A., OYASU, R., PREISINGER, A.C., VOGELSTEIN, B. (1992a). Clonal origin of bladder cancer. *New Engl. J. Med.*, **326**, 737-740
- SIDRANSKY, D., MIKKELSEN, T., SCHWECHHEIMER, K., ROSENBLUM, M.L., CAVANEE, W., VOGELSTEIN, B. (1992b). Clonal expansion of p53 mutant cells is associated with brain tumour progression. *Nature*, **355**, 846-847
- SMITH, P.G., KINLEN, L.J., WHITE, G.C., ADELSTEIN, A.M., FOW, A.J. (1980). Mortality of wives of men dying with cancer of the penis. *Br. J. Cancer*, **41**, 422-428
- SMITH, T.A., WHELAN, J., PARRY, P.J. (1992). Detection of single-base mutation in a mixed cell population of cells: a comparison of SSCP and direct sequencing. *GATA*, **9**, 143-145
- SNIJDERS, P.J.F., MEIJER, C.J.L.M., WALBOOMERS, J.M.M. (1991). Degenerate primers based on highly conserved regions of amino acid sequence in papillomaviruses can be used in a generalised polymerase chain reaction to detect productive human papillomavirus infections. *J. Gen. Virol.*, **72**, 2781-2786

- SOLER, C., CHARDONNET, Y., EUVRARD, S., CHIGNOL, M.C., THIVOLET, J. (1992). Evaluation of human papillomavirus type 5 on frozen sections of multiple lesions from transplant recipients with in situ hybridisation and non-isotopic probes. *Dermatology*, **184**, 248-253
- SOUSA, R., DOSTATNI, N., YANIV, M. (1990). Control of papillomavirus gene expression. *Biochemica et Biophysica acta.*, **1032**, 19-37
- SOUSSE, T., CARON DE FROMENTEL, C., MECHALI, M., MAY, P., KRESSI, M. (1987). Cloning and characterisation of a cDNA from *Xenopus Laevis* coding for a protein homologous to human and murine p53. *Oncogene*, **1**, 71-78
- SOUSSE, T., CARON, C., DE FROMENTAL., MAY, P. (1990). Structural aspects of the P53 protein in relation to gene evolution. *Oncogene*, **5**, 945-952.
- SPALHOLZ, B.A., YANG, Y.C., HOWLEY, P.M. (1985). Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. *Cell*, **42**, 183-191
- SPALHOLZ, B.A., McBRIDE, A.A., SARAFI, T., QUINTERO, J. (1993). Binding of bovine papillomavirus E1 to the origin is not sufficient for DNA replication. *Virology*, **193**, 201-212
- SPALHOLZ, B.A. (1993). Importance of the papillomavirus P2443 promoter in the regulation of E2 and E5 expression. *J. Virol.*, **67**, 6278-6284
- STANLEY, M (1990). Genital papillomaviruses, polymerase chain reaction and cervical cancer. *Genitourin Med*, **66**, 415-417.
- STEELE, J.C., STANKOUK, T., GALLIMORE, P.H. (1993). Production and characterization of human proliferation T cell clones specific for human papillomavirus type 1 E4 protein. *J. Virol.*, **67**, 2799-2806
- STEGER, G., PFISTER, H. (1992). In vitro expressed HPV8 E6 protein does not bind p53. *Arch. Virol.*, **125**, 355-360
- STEINMEYER, K., MAACKKE, H., DEPPERT, W. (1990). Cell cycle control by p53 in normal (3T3) and chemically transformed (Meth A) mouse cells. 1. Regulation of p53 expression. *Oncogene*, **5**, 1691-1699.
- STENGER, J., MAYR, G., MANN, K., TEGTMEYER, P. (1992). Formation of stable p53 homotetramers and multiples of tetramers. *Mol. Carcinog.*, **5**, 102-105
- STERLING, J., STANLEY, M., GATWARD, G., MINSON, T. (1990). Production of human papillomavirus type 16 virions in a keratinocyte cell line. *J. Virol.*, **64**, 6305-6307



- STINGL, G., STINGL, L.A.G., ABERER, W., WOLFF, K. (1981). Antigen presentation by murine epidermal Langerhans cells and its alteration by ultraviolet B light. *J. Immunol.*, **127**, 1707-1713
- STONE, M., NOONAN, C., TSCHEN, J. (1987). Bowens disease of the feet: presence of human papillomavirus 16 DNA in tumour tissue. *Arch. Dermatol.*, **123**, 1517-1520
- STOREY, A., PIM, D., MURRAY, A., OSBORN, K., BANKS, L., CRAWFORD, L. (1988). Comparison of the in vitro transforming activities of HPV types. *EMBO. J.*, **7**, 1815-1820
- STRATTON, M.R. (1992). The P53 gene in human cancer. *Eur. J. Cancer*, **28**, 293-295.
- STREILEIN, J.W. (1991). Immunogenic factors in skin cancer. *New Engl. J. Med.*, **325**, 884-886
- STRIVASTAVA, S., ZOU, Z., PIROLLO, K., BLATTNER, W., CHANG, E.H. (1990). Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature*, **348**, 747-749
- STURZBECHER, H.W., MAIMETS, T., CHUMAKOV, P., BRAIN, R., ADDISON, C., SIMANIS, V., RUDGE, K., PHILIP, R., GRIMALDI, M., COURT, W., JENKINS, J.R. (1990). p53 interacts with p34cdc2 in mammalian cells- implications for cell cycle control and oncogenesis. *Oncogene*, **5**, 795-802
- TAKAHASHI, T., NAU, M.M., CHIBA, I., BIRRER, M.J., ROSENBERG, R.K., VINOCOUR, M., LEVITT, M., PASS, H., GAZDAR, A.F., MINNA, J.D. (1989). p53 - a frequent target for genetic abnormalities in lung cancer. *Science*, **246**, 491-494
- TAKAHASHI, T., CORBONE, D., TAKAHASHI, T., NAU, M.M., HIDA, T., LINNOILA, I., VEDA, R., MINNA, J.D. (1992). Wild type but not mutant p53 suppresses the growth of human lung cancer cells bearing multiple genetic lesions. *Cancer Res.*, **52**, 2340-2343
- TAKAMI, Y., SASGAWA, T., SUDIRO, T.M., YUTSUDO, M., HAKURA, H. (1992). Determination of the functional difference between human papillomavirus type 6 and 16 E7 proteins by their 30 N-terminal amino acid residues. *Virology*, **186**, 489-495
- TAMURA, G., KIHANA, T., NOMURA, K., TERADA, M., SUGIMURA, T., HIROBASHI, S. (1991). Detection of frequent p53 gene mutations in primary gastric cancer by cell sorting and polymerase chain reaction single strand conformational polymorphism analysis. *Cancer Res.*, **51**, 3056-3058



- TARUNINA, M., JENKINS, J.R. (1993). Human p53 binds DNA as a protein homodimer but monomeric variants retain transcription transactivation activity. *Oncogene*, **8**, 3165-3173
- THEIRRY, F., YANIV, M. (1987). The BPV-1-E2 trans-acting protein can be either an activator or a repressor of the HPV 18 regulatory region. *EMBO. J.*, **6**, 3391-3397
- THIELMANN, H.W., EDLER, L., BURKHARDT, M.R., JUNG, E.G. (1987). DNA repair synthesis in fibroblast strains from patients with actinic keratoses, squamous cell carcinoma, basal cell carcinoma or malignant melanoma after treatment with ultraviolet light, N-acetoxy-2-acetyl aminofluorene, methyl methane sulphonate and W-methyl-N-nitrosourea. *J.Cancer Res. Clin. Oncol.*, **113**, 171-186
- THOMSON, J.F., ALLEN, R., MORRIS, P.J., WOOD, R. (1985). Skin cancer in renal transplant patients treated with cyclosporin. *Lancet*, **I**, 158
- THORNER, L.K., LIM, D.A., BOTCHAN, M.R. (1993). DNA binding domain of bovine papillomavirus type 1 E1 helicase: structural and functional aspects. *J. Virol.*, **67**, 6000-6014
- TIEBEN, M.L., BERKHOUT, R.J.M., SMITS, H.L., BAVINCK, J.N.B., VERMEER, B.J., BRUIJN, J.A., VANDERWOUDE, F.J., TERSCHEGGET, J. (1994). Detection of epidermodysplasia verruciformis-like human papillomavirus types in malignant and pre-malignant skin lesions of renal transplant recipients. *Br. J. Dermatol.*, **131**, 226-230
- TILBROOK, P.A., GREENOAK, G.E., KULSKI, J.K. (1989). Identification of papillomaviral DNA sequences in hairless mouse-tumours induced by ultraviolet radiation. *J. Gen. Virol.*, **70**, 1005-1009
- TING, Y., MANOS, M. (1990). Detection and typing of genital human papillomaviruses. in *PCR Protocols; A Guide To Methods And Applications*. pp356-367
- TOMMASINO, M., ADAMCZEWSKI, J.P., CARLOTTI, F., BARTH, C.F., CONTORNI, M., CAVALIERI, F., HUNT, T., CRAWFORD, L. (1993). HPV16 E7 protein associates with the protein kinase p33<sup>cdk2</sup> and cyclin A. *Oncogene*, **8**, 195-202
- TSAI, Y.C., NICHOLAS, P.W., HITI, A.L., WILLIAMS, Z., SKINNER, D.J., JONES, P.A. (1990). Allelic losses of chromosomes 9, 11, and 17 in human bladder cancer. *Cancer Res.*, **50**, 44-47
- TSUKADA, T., TOMOOKA, Y., TAKAI, S., VEDA, Y., NISHIKAWA, S., YAGE, T., TOKUNAGA, T., TAKEDA, N., SUDA, Y., ABE, S., MATSUO, I., KAWA, Y., AIZAWA, S. (1993). Enhanced proliferative potential in culture cells from p53 deficient mice. *Oncogene*, **8**, 3313-3322

- ULLRICH, S.E., KRIPKE, M.L. (1984). Mechanisms in the suppression of tumour rejection produced in mice by repeated UV radiation. *J. Immunol.*, **133**, 2786-2790
- UNGER, T., NAU, M.N., SEGAL, S., MINNA, J.D. (1992). p53: a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. *EMBO. J.*, **11**, 1383-1390
- VAN DER LEEST, R.J., ZACHOW, K.R., OSTROW, R.S., BENDER, M., PASS, F., FARAS, A.J. (1987). Human papillomavirus heterogeneity in 36 renal transplant recipients. *Arch. Dermatol.*, **123**, 354-357
- VARLEY, J.M., BRAMMER, W.J., LANE, D.P., SWALLOW, J.E., DOLAN, C., WALKER, R.A. (1991). Loss of chromosome 17p13 sequences and mutation in human breast cancer carcinomas. *Oncogene*, **6**, 413-421
- VITALLIANO, P.P., URBACH, F. (1980). The relative importance of risk factors in nonmelanoma carcinoma. *Arch. Dermatol.*, **116**, 454-456
- VOGELSTEIN, B., FEARON, E.R., HAMILTON, S.R., KERN, S.E., PREISINGER, A.C., LEPPERT, M., NAKAMURA, Y., WHITE, R., SMITS, A.M.M., BOS, J.I. (1988). Genetic alterations during colorectal-tumor development. *New Engl. J. Med.*, **319**, 525-532
- VOGELSTEIN, B., KINZLER, K.W. (1992a). P53 Function and dysfunction. *Cell*, **70**, 523-526.
- VOGELSTEIN, B., KINZLER, K.W. (1992b). Carcinogens leave fingerprints. *Nature*, **355**, 209-210
- VOGELSTEIN, B., KINZLER, W. (1994). X-rays strike p53 again. *Nature*, **370**, 174-175
- VOJTESEK, B., BARTEK, J., MIDGLEY, C.A., LANE, D.P. (1992). An immunochemical analysis of the human nuclear phosphoprotein-p53-new monoclonal-antibodies and epitope mapping using recombinant-p53. *J. Immunol. Meth*, **151**, 237-244
- VOJTESEK, B., LANE, D.P. (1993). Regulation of p53 protein expression in human breast cancer cell lines. *J. Cell Science*, **105**, 607-612.
- VOUSDEN, K. (1993). Interactions of human papillomavirus transforming proteins with the products of tumour suppressor genes. *FASEB. J.*, **7**, 872-879.
- WALDECK, W., ROSI, I., ZENTGRAF, H. (1984). Origin of replication in episomal bovine papillomavirus type-1 DNA isolated from transformed cells. *EMBO. J.*, **6**, 3391-3397

- WANK, R.E., THOMSEN, C. (1991). High risk of squamous cell carcinoma of the cervix for women with HLA-Dqw3. *Nature*, **352**, 723-724
- WARD, P., COLEMAN, D.V., MALCOLM, A.D.B. (1989). Regulatory mechanisms of the papillomaviruses. *Trends In Genetics*, **5**, 97-99
- WATTS, S.L., PHELPS, W.C., OSTROW, R.S., ZACHOW, K.R., FARAS, A.J. (1984). Cellular transformation by human papillomavirus DNA in vitro. *Science*, **225**, 634-636
- WEINTRAUB, H., HAUSCHKA, S., TAPSCOTT, S.J. (1991). The mck enhancer contains a p53 responsive element. *Proc. Natl. Acad. Sci. USA.*, **88**, 4570-4571
- WERNES, B.A., LEVINE, A.J., HOWELY, P.M. (1990). Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science*, **248**, 76-79
- WESTON, A., WILLEY, J.C., MODALI, R., SIGIMURA, H., MCDOWELL, E.M., RESAU, J., LIGHT, B., HAUGEN, A., MANN, D.L., TRUMP, B.F., HARRIS, C.C. (1989). Differential DNA sequence deletions from chromosomes 3, 11, 13 and 17 in squamous-cell carcinoma, large-cell carcinoma and adenocarcinoma of the human lung. *Proc. Natl. Acad. Sci. USA.*, **86**, 5099-5103
- WILLIAMS, G.T., SMITH, C.A. (1993). Molecular regulation of apoptosis: Genetic controls on cell death. *Cell*, **74**, 777-779
- WILSON, C.A.B., HOLMES, S.C., CAMPO, M.S., WHITE, S.I., TILLMAN, D., MACKIE, R.M. THOMSON, J. (1989). Novel variants of human papillomavirus type 2 in warts from immunocompromised individuals. *Br. J. Dermatol.*, **121**, 571-575
- WU, J.K., YE, Z., DARRAS, B.T. (1993). Sensitivity of single-strand conformation polymorphism (SSCP) analysis in detecting p53 point mutations in tumours with mixed cell populations. *Am. J. Hum. Genet.*, **52**, 1273-1275
- WYNFORD-THOMAS, D. (1992). p53 in tumour pathology: can we trust immunocytochemistry? *J. Pathol.*, **166**, 329-330
- YABE, Y., TANIMURA, Y., SAKIA, A., HITSUMOTO, T., NOHARA, N. (1989). Molecular characteristics and physical state of human papillomavirus DNA change with progressing malignancy: studies in a patient with epidermodysplasia verruciformis. *Int. J. Cancer*, **43**, 1022-1028
- YAMASHITA, T., SEGAVVA, K., FUJINAGA, Y., NISHIKAWA, T., FUJINAGA, K., (1993). Biological and biochemical activities of E7 genes of the cutaneous human papillomavirus type 5 and 8. *Oncogene*, **8**, 2433-2441.

- YANG, Y.C., OKAYAMA, H., HOWLEY, P.M. (1985). Bovine papillomavirus contains multiple transforming genes. *PNAS*, **82**, 1030-1034
- YEW, P.R., BERK, A.J. (1992). Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature*, **357**, 82-85
- YEWDELL, J.W., GANNON, J.V., LANE, D.P. (1986). Monoclonal antibody analysis of p53 expression in normal and transformed cells. *J. Virol*, **59**, 444-452
- YIN, Y., TAINSKY, M.A., BISCHOTT, F.Z., STRONG, L.C., WAHL, G.M. (1992). Wild type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell*, **70**, 937-948
- YONISH-ROUACH, E., RESNITZKY, D., LOTEM, J., SACHS, L., KIMCHI, A., OREN, M. (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature*, **352**, 345-347
- YOSHIKAWA, T., RAE, U., BRUINS-SLOTT, W. (1990). Susceptibility to effects of UV radiation on induction of contact hypersensitivity as a risk factor for skin cancer in humans. *J. Invest. Dermatol*, **95**, 530-536
- YUSPA, S.H., MORGAN, D.L. (1981). Mouse skin cells resistant to terminal differentiation associated with initiation and carcinogenesis. *Nature*, **293**, 72-74
- YUSPA, S.H., KILKENNY, A.E., STANLEY, J., LICHTI, U. (1985). Keratinocytes blocked in phorbol ester-responsive early stage of terminal differentiation by Sarcoma viruses. *Nature*, **314**, 459-462
- YUSPA, S.H., MORGAN, D., LICHTI, U., SPANGLER, E.F., MICHAEL, D., KILKENNY, A., HENNINGS, H. (1986). Cultivation and characterisation of cells derived from mouse skin papillomas induced by an initiation-promotion protocol. *Carcinogenesis*, **7**, 949-958
- YUSPA, S.H., KILKENNY, A.E., CHENG, C., ROOP, D., HENNINGS, H., KRUSZEWSKI, F., LEE, E., STRICKLAND, J., GREENHALGH, D.A. (1991). Alterations in epidermal biochemistry as a consequence of stage-specific changes in skin carcinogenesis. *Environ. Health Prospect.*, **93**, 3-10
- YUTSUDO, M., OKAMOTO, Y., HAKURA, A. (1988). Functional dissociation of transforming genes of human papillomavirus type 16. *Virol.*, **166**, 594-597
- ZACHOW, K.R., OSTROW, R.S., FARAS, A.J. (1987). Nucleotide sequence and genome organization of human papillomavirus type 5. *Virology*, **158**, 251-254
- ZAMBETTI, G.P., OLSON, D., LABOW, M., LEVINE, A.J. (1992). A mutant p53 protein is required for the maintenance of the transformed phenotype in p53 plus ras transformed cells. *Proc. Natl. Acad. Sci. USA*. **89**, 3952-3956



ZAMBETTI, G.P., LEVINE, A.J. (1993). A comparison of the biological activities of wild type and mutant p53. *FASEB*, **7**, 855-865

ZIEGLER, A., LETTELL, D.J., KUNALA, S., SHARMA, H.W., GUILANI, M., SIMON, J.A., HALPERIN, A.J., BADEN, H.P., SHAPIRO, P.E., BALE, A.E., BRASH, B.E. (1993). Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. *Proc. Natl. Acad. Sci. USA.*, **90**, 4216-4220

ZUR HAUSEN, H. (1982). Human genital cancer; Synergism between two virus infections or synergism between a virus infection and initiating events. *Lancet* **II**, 1370-1372.

ZUR HAUSEN, H. (1989). Papillomaviruses in anogenital cancer as a model to understand the role of viruses in human cancers. *Cancer Res.*, **49**, 4677-4681.

ZUR HAUSEN, H. (1991). Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* **184**, 9-13.



## **APPENDIX 1:**

**APPENDIX 1: ASSOCIATION BETWEEN RENAL ALLOGRAFT  
RECIPIENT PATIENT CODES AND SAMPLE NUMBERS**

<b>Patient Code</b>	<b>Transplant Duration (yrs)</b>	<b>Sample Number</b>	<b>Histology</b>
<b>1</b>	15	98	US
<b>2</b>	13	140	VW
<b>3</b>	18	51	VW(D+)
		142	US
<b>4</b>	9	172	US
		165	MISC
<b>5</b>	8	121	US
<b>6</b>	2	119	US
		159	MISC
		192	IEC
		193	SCC
<b>7</b>	13	211	US
<b>8</b>	6	102	IEC
		173	AK (D+++)
		174	AK(D+++)
<b>9</b>	12	118	US
<b>10</b>	8	195	US
<b>11</b>	9	182	VW
<b>12</b>	15	9	VK(D+)
		10	AK(D+++)
		53	VW
		189	SCC (in VK)
		215	VK
		216	VK
		217	VK
		219	VK
<b>13</b>	5	198	IEC
<b>14</b>	4	89	IEC
		97	US
<b>16</b>	17	1	SCC
		60	AK
		105	US
		131	SCC
		147	SCC
		148	AK(D+)
		149	IEC
		158	VK

Patient Code	Duration Of Transplant (yrs)	Sample Code	Histology
16	17	163	SCC
		181	VK
		183	IEC
		184	IEC
		188	SCC
		196	MISC
		209	SCC
17	12	202	US
18	6C	42	AK(D++)
		68	VK
		69	AK
19	1	157	AK(D+++)
20	21	28	IEC
		85	VK
		86	VW
		92	SCC
		96	SCC
		106	SCC
		122	SCC
		123	IEC
		133	IEC
		134	IEC
		155	SCC
		156	SCC
		166	AK
		185	SCC
		186	SCC
		187	SCC
21	23	128	US
22	14	141	US
23	23	7	VW
		8	VW
		38	BCC
		39	AK
		91	IEC
		103	IEC
		104	AK(D+)
		124	IEC
		136	VW
		138	IEC
		139	SCC
		162	VW
		190	IEC

<b>Patient Code</b>	<b>Duration Of Transplant (yrs)</b>	<b>Sample Code</b>	<b>Histology</b>
<b>23</b>	<b>23</b>	191	IEC
		200	AK
		201	IEC
		205	AK(D+)
<b>24</b>	<b>2</b>	178	US
<b>25</b>	<b>8</b>	151	MISC
		152	MISC
<b>26</b>	<b>10</b>	2	SCC
		3	VK(D+)
		4	IEC
		5	IEC
		6	VK(D+)
		11	SCC
		12	AK(D+)
		16	VW(D+)
		17	VW
		26	VW
		26A	IEC
		27	AK(D+)
		36	VK(D+)
		48	IEC
		49	SCC
		54	SCC
		55	SCC
		71	SCC
		72	SCC
<b>27</b>	<b>8</b>	75	IEC
		76	IEC
		111	AK
		112	US
<b>28</b>	<b>9</b>	154	VW
<b>29</b>	<b>3</b>	46	VW
		47	VW
<b>30</b>	<b>14</b>	108	VW
<b>31</b>	<b>11</b>	88	VW
		115	VW
<b>32</b>	<b>3</b>	57	VW
<b>33</b>	<b>9</b>	58	MISC
		59	MISC
		113	MISC
		114	MISC
<b>34</b>	<b>26</b>	32	IEC
		33	AK(D+)

Patient Code	Duration Of Transplant (yrs)	Sample Code	Histology
34	26	34	VW(D+)
		35	IEC
		37	SCC
		66	SCC
		67	SCC
		77	SCC
		100	IEC
		101	SCC
		125	SCC
		126	SCC
		127	SCC
35	14	94	MISC
		95	AK
36	7	120	US
37	3	203	VW
38	3	204	MISC
39	9C	132	SCC
40	13	40	IEC
		41	AK
		150	VK
		160	US
41	21	164	US
42	12	107	US
43	7	81	VW
44	13	207	AK(D+)
		208	AK(D+)
45	9	218	VW
46	16	135	US
47	5C	43	VW
48	4	129	BCC
		130	BCC
		161	US
		206	BCC
49	25	50	AK(D+)
50	8	73	VK(D+)
		79	AK
		80	AK(D++)
51	3	87	MISC
		117	US
52	9	199	AK(D+)
53	6	29	IEC
		30	VW



Patient Code	Duration Of Transplant (yrs)	Sample Code	Histology
54	15	13	VK(D+)
		14	AK
		31	BCC
		109	US
		110	VK
		143	IEC
		144	VK(+++)
		179	SCC
		197	VK(D+)
		210	SCC
		212	IEC
		213	SCC
55	11	176	VW
		177	US
56	10	153	MISC
57	15	62	VK
		62A	VK(D+)
58	10	93	SCC
59	1	175	BCC
		214	IEC
60	20	180	VW
61	15	194	VK(D+)
62	13	15	BCC
63	3	63	VW
64	12	18	AK(D+)
		19	MISC
		20	IEC
		21	SCC
		22	SCC
		23	IEC
		24	IEC
		25	SCC

yrs= years; C = cardiac transplant recipient; VW = viral wart; VK = verrucous keratosis; AK = actinic keratosis; D = mild dysplasia; D++ = moderate dysplasia; D+++ = severe dysplasia; IEC = intraepidermal carcinoma; SCC = squamous cell carcinoma; BCC = basal cell carcinoma; MISC = miscellaneous lesions.

**APPENDIX 2:-**  
**PUBLICATIONS**

# Prevalence of human papillomavirus DNA in cutaneous neoplasms from renal allograft recipients supports a possible viral role in tumour promotion

L.A. Stark<sup>1</sup>, M.J. Arends<sup>1</sup>, K.M. McLaren<sup>1</sup>, E.C. Benton<sup>2</sup>, H. Shahidullah<sup>2</sup>, J.A.A. Hunter<sup>2</sup> & C.C. Bird<sup>1</sup>

Departments of <sup>1</sup>Pathology and <sup>2</sup>Dermatology, University of Edinburgh, Edinburgh, UK.

**Summary** It is well established that renal allograft recipients (RARs) have an increased incidence of viral warts and premalignant and malignant cutaneous lesions, and the risk of their development increases in proportion to duration of graft survival. It has been postulated that, in addition to the effects of prolonged immunosuppression and previous sun exposure, human papillomaviruses (HPV) may also contribute to the carcinogenic process. In this study, the prevalence of HPV DNA was examined in a range of premalignant and malignant cutaneous tumours from 50 immunosuppressed patients (47 renal allograft recipients plus three cardiac allograft recipients) and 56 immunocompetent patients using Southern hybridisation as a low-stringency screening method and type-specific polymerase chain reaction (PCR) assays for eight HPV types. The combined results for renal allograft recipients show that HPV DNA was detectable in 79% of viral warts, 42% of premalignant keratoses, 33% of intraepidermal carcinomas, 43% of invasive squamous cell carcinomas and 16% of uninvolved skin specimens (squamous cell carcinomas/renal allograft recipients significantly different at  $P < 0.05$  from uninvolved skin specimens/renal allograft recipients). In immunocompetent patients the pattern of HPV DNA prevalence was 100% for viral warts; 25% for keratoses, 23% for intraepidermal carcinomas, 22% for squamous cell carcinomas and 8% for uninvolved skin. No single HPV type predominated in tumour specimens from either group. More tumours were found to contain HPV DNA by Southern hybridisation analysis than PCR, indicating the presence of HPV types other than HPV 1, 2, 5, 6, 8, 11, 16 and 18 in some tumours. However, 'low cancer risk' HPV types 1, 2 and 6 as well as 'high cancer risk' HPV types 5 and 16 were specifically detected by PCR in a small number of neoplasms. These data suggest that multiple HPV types may contribute to cutaneous neoplasia in RARs and that they appear to act early in the process of carcinogenesis, perhaps by functioning as tumour promoters via stimulation of cell proliferation.

Renal transplantation is now a well-established procedure, with many recipients surviving 20 years or more. However, a major problem associated with long-term immunosuppression is the increased prevalence of various malignancies, especially in skin, anogenital tract and lymphoreticular system (Hoxell *et al.*, 1977; Birkeland, 1983; Blohme & Larko, 1984; Sheil *et al.*, 1985; Shuttleworth *et al.*, 1987; Alloub *et al.*, 1989). Moreover, renal allograft recipients (RARs) frequently develop a spectrum of cutaneous complications ranging from benign viral warts (VWs), to verrucous and actinic keratoses (Ks) exhibiting varying degrees of dysplasia, culminating in squamous cell carcinoma (SCC) (Benton *et al.*, 1992). The prevalence and morbidity of such complications increases the longer the duration of immunosuppression with a number of long-standing RARs developing multiple skin tumours (Barr *et al.*, 1989). In RARs squamous cell cancers outnumber basal cell cancers (BCCs) by a ratio of 15:1, a reversal of the 1:5 ratio normally observed in immunocompetent patients.

A number of factors have been implicated in the development of skin cancers in RARs. Ultraviolet (UV) radiation is known to be of considerable importance as the majority of tumours occur on sun-exposed skin (Blohme & Larko, 1984; Boyle *et al.*, 1984; Baadsgaard, 1991; Streilein, 1991). The alteration in cell-mediated immunity brought about by prolonged immunosuppressive therapy is thought to be a contributory factor and is associated with an increased incidence of anogenital cancers and lymphomas as well as skin tumours (Streilein, 1991). The possible association with human papillomaviruses (HPVs) is derived indirectly from observations in the rare, inherited skin disease epidermodysplasia verruciformis (EV) (Orth *et al.*, 1979; Orth, 1986). This disease is characterised by the development of extensive,

persistent infection with unusual HPV types and a predisposition to cutaneous SCC on light-exposed skin in around one-third of patients (Pfister *et al.*, 1983a; Fuchs & Pfister, 1990). Although over 20 HPV types have been detected in benign skin lesions from EV patients, in SCC HPV types 5 and 8 are consistently demonstrated (Orth *et al.*, 1986; Fuchs & Pfister, 1990). However, in contrast to the HPV types 16 and 18 that are usually integrated in squamous cervical cancers, the majority of EV-associated SCCs contain HPV 5 or 8 DNA in an episomal form, with integration being a rare event (Yabe *et al.*, 1989).

There is also some direct evidence to suggest that HPV may play a part in the development of skin cancers in RARs (Blessing *et al.*, 1989; Benton *et al.*, 1992). Histologically, viral warts and keratotic lesions in RARs often exhibit varying degrees of epidermal dysplasia, while SCCs develop on a background of verrucous keratoses and may retain HPV-associated features. However, the detection of HPV DNA in the cutaneous SCC of RARs has been somewhat controversial, with EV-associated types and a variety of common cutaneous and genital HPV types being identified in some, but not all, studies (Lutzner *et al.*, 1980; Van der Leest *et al.*, 1987; Barr *et al.*, 1989; Rudlinger & Grob, 1989; Dyall-Smith *et al.*, 1991; Soler *et al.*, 1992).

We report here the results of an investigation in which we determined, first, the prevalence of HPV DNA in various cutaneous lesions from RARs and immune-competent patients (ICPs) by Southern hybridisation analysis with mixed probes for common cutaneous and EV-associated HPV types. Second, using type-specific and sensitive PCR assays we determined the prevalence of the putative oncogenic HPV types 5 and 8, the more common cutaneous HPV types 1 and 2 and the common genital HPV types 6, 11, 16 and 18. Finally, we considered whether the pattern of HPV prevalence in the cutaneous lesions provided clues as to the stage at which HPV may act in the oncogenic process.

## Materials and methods

### Patients

Two groups of patients were investigated. The first comprised 47 immunosuppressed patients, 44 RARs plus three cardiac allograft recipients (mean age 50 years, range 20–71 years), all of whom received transplants between 1965 and 1992 (mean duration of transplant 10.9 years, range 1–26 years). Prior to 1984 patients received immunosuppressive therapy with prednisolone and azathioprine, but since then all new allograft recipients have been treated with prednisolone and cyclosporin A, a few subsequently being switched to azathioprine. The second patient group comprised 56 immunocompetent individuals (mean age 66.6 years, range 22–90 years) who were referred for treatment of suspected warts or skin malignancies. All patients were treated in the Department of Dermatology at the Royal Infirmary of Edinburgh.

### Tissue collection and DNA extraction

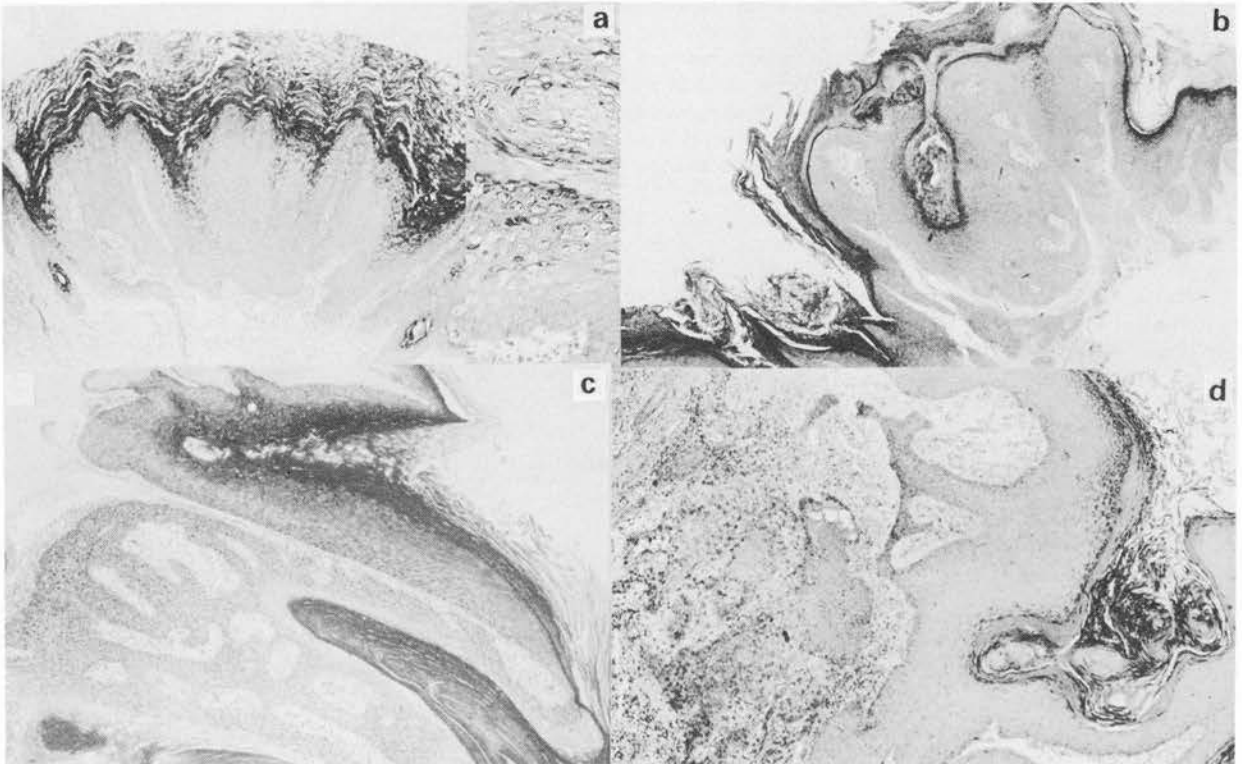
Therapeutic skin biopsies were collected from RARs (120 in all) and ICPs (63). A 6 mm biopsy of uninvolved, sun-exposed, forearm skin was also obtained from 19 RARs (some with and others without skin tumours elsewhere) and 12 healthy ICPs who volunteered to undergo this procedure. Immediately following excision, each lesion was bisected longitudinally with a sterile blade to minimise the risk of contamination: half was snap frozen in liquid nitrogen prior to DNA extraction while the remainder was fixed in formalin or periodate–lysine–paraformaldehyde–dichromate (PLPD) (Holgate *et al.*, 1986) for histological examination. Frozen tissue was minced in lysis buffer (50 mM Tris, 50 mM EDTA, 100 mM sodium chloride, 5 mM DTT, 1% SDS, 1.5 mg ml<sup>-1</sup> proteinase K) and DNA extracted using a standard phenol–chloroform extraction technique (Sambrook *et al.*, 1989).

### Histopathology

The cutaneous lesions were assessed for standard morphological features suggestive of actinic damage and for degrees of dysplasia progressing to intraepidermal and invasive carcinoma (Blessing *et al.*, 1989). They were designated as viral warts (VWs), actinic and verrucous keratoses (AKs and VKs), intraepidermal carcinoma (IEC) and squamous cell carcinoma (SCC) (Figure 1). VVs showed architectural symmetry, hypergranulosis and koilocytosis. Lesions showing double-layered basal budding, basal hypermelanosis and dysplasia and loss of granular layer with superficial parakeratosis were classified as actinic keratoses. Lesions that showed some features suggestive of HPV infection, but various degrees of basal budding and basal dysplasia, were termed verrucous keratoses. IECs exhibited either full-thickness dysplasia or severe dysplasia in the basal layer. The designation of SCC was confined to lesions in which there was evidence of dermal invasion. In some instances, the complex architecture of VKs and the variable dysplasia made confirmation of invasion difficult so the term SCC was used only when dermal invasion was unequivocal (Blessing *et al.*, 1989).

### Polymerase chain reaction (PCR)

Oligonucleotide primers, situated in E6, were designed from published sequence data (Danos *et al.*, 1982; Fuchs *et al.*, 1986; Zachow *et al.*, 1987; Hirsch-Behnam, 1990) to detect HPV types 1, 2, 5 and 8 in type-specific assays (Table I). Primer sequences for HPV types 6, 11, 16 and 18 were validated in previous studies (Arends *et al.*, 1991). Prior to amplification with HPV primers, each sample was amplified with control *ras* primers to confirm adequate preservation of DNA (Table I). A 1 µg aliquot of genomic DNA was used as template in a 100 µl reaction containing 1 × preprepared reaction buffer (NBL), 200 µM dNTPs 1 µM each primer and 0.5 U of *Taq* polymerase (NBL). PCR cycle conditions used



**Figure 1** a, Viral wart exhibiting papilliferous architecture. Inset shows cell vacuolation (koilocytotic change) and cytoplasmic inclusions at high power. b, Verrucous keratosis with the topography of a viral wart but lacking the cytological features. There is some irregularity of the basal tongues. c, Verrucous keratosis with widespread dysplasia amounting to intraepidermal carcinoma. d, Invasive squamous cell carcinoma arising from a surface exophytic verrucous keratosis (haematoxylin and eosin).



**Table 1** HPV primer sequences used to detect HPV types 1, 2, 5 and 8, and K-ras

HPV type	Primer	Sequence	Position <sup>a</sup>	Product
1	p1	AGTCTTATGAGGTACCGGAAATAGAAG	383–409	
1	p2	ATGCACTCTTTCTCCGTTTGACACAACCTC	520–490	136 bp
2	p1	ATGGTTTGGAGCTAGAGGATTTGCG	159–183	
2	p2	AACTAGTAATGCCTCCTTCTCCTCC	463–438	303 bp
5	p1	CTCTAATACCAAATTCTGTGGCGT	616–640	
5	p2	GAGGAACGCCTGGAAGGGAATCTG	894–870	279 bp
8	p1	CGGGCAGGACAAGGCTTCATATTTAGACAC	200–230	
8	p2	ACAACAACGACAACACGCAGTAACAAC	420–393	220 bp
K-ras	p1	GACTGAATATAAACTTGTGG	3–22	
K-ras	p2	CTCTATTGTTGGATCATATT	111–92	109 bp

<sup>a</sup>Position in HPV genome defined by EMBL/Genbank database.

to amplify HPV types 1, 2, 5 and 8 were as follows: One cycle of 94°C for 5 min; 30 cycles of 58°C (55°C for HPV 1) for 2 min, 72°C for 3 min and 94°C for 1 min; and one cycle of 58°C (55°C for HPV 1) for 2 min and 72°C for 10 min. Positive (1 pg of purified HPV plasmid DNA instead of genomic DNA) and negative (template-free) controls were included with all reactions. Amplified products were visualised on a 2% Nusieve-Seakem (3:1) agarose gel containing 0.5 µg ml<sup>-1</sup> ethidium bromide (Flowgen Instruments, Kent, UK).

#### Southern hybridisation analysis

Genomic DNA (8–10 ng) was digested using the restriction enzyme *Bam*HI (NBL). Following electrophoresis on 8% agarose gel, DNA was alkaline denatured and transferred on to charged nylon membrane (Hybond N<sup>+</sup>, Amersham, Aylesbury, UK) according to the manufacturer's instructions. HPV probe DNA was isolated from vector DNA by digestion with the appropriate restriction enzyme followed by electrophoresis on a 0.8% low melting temperature agarose gel. The resulting HPV DNA was purified using Biorad Prepagene kit (Biorad Laboratories, Richmond, UK) and 25 ng DNA of each HPV type was prepared and labelled with <sup>32</sup>P using the Amersham Multiprime kit as specified by the manufacturer's instructions. Hybridisation was carried out at *T*<sub>m</sub> – 40°C (55°C) (hybridisation buffer consisted of 6 × SSC, 1% SDS and 0.1 g ml<sup>-1</sup> dextran sulphate) using mixed HPV probes containing 25 ng each of either HPV types 3, 8 and 13 or HPV types 2, 4 and 12. The minimum specific activity of all probes was 4–5 × 10<sup>6</sup> c.p.m. ml<sup>-1</sup>. All filters included positive (HPV plasmid DNA) and negative (placental DNA) controls. Following hybridisation, filters were washed at low stringency [2 × SSC, 1% SDS, at 55°C for 30 min (*T*<sub>m</sub> – 35°C)] and exposed to X-ray film initially for 24 h and subsequently for 3 days. Cases positive for HPV by this initial screen were further analysed by Southern hybridisation using the restriction enzyme *Pst*I. Filters containing 50 pg of purified HPV plasmid DNA of types 1, 2, 3, 4, 5, 8, 10, 12, 13, 14, 17, 19 and 20 (Heilman *et al.*, 1980; Ostrow *et al.*, 1982, 1983; Kremsdorf *et al.*, 1983, 1984; Pfister *et al.*, 1983a, b; Gassenmaier *et al.*, 1984) mixed with 10 µg of genomic DNA were also made for use in initial optimisation experiments. The sensitivity of this technique was investigated by performing Southern hybridisation analyses on serially diluted HPV 16 plasmid DNA mixed with a known concentration of genomic DNA.

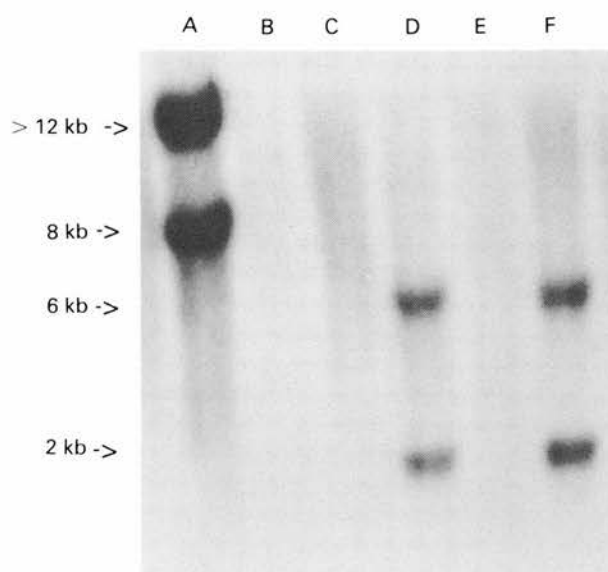
## Results

#### Prevalence screen for HPV DNA by Southern hybridisation

Initial experiments indicated that, by using Southern hybridisation with a probe cocktail containing a mixture of HPV types 3, 8 and 13 at low hybridisation (*T*<sub>m</sub> – 40°C) and washing (*T*<sub>m</sub> – 35°C) stringency, it was possible to detect

HPV types 1, 2, 3, 4, 5, 8, 10, 12, 13, 14, 17, 19 and 20. The probe cocktail containing HPV types 2, 4 and 12 was also extensively used, but added little extra information. The sensitivity of Southern hybridisation analysis was found to be 5 pg of viral DNA in a background of 10 µg of genomic DNA, equivalent to 0.1 copies per cell.

A total of 108 skin biopsies from RARs, including over 50 IEC and SCC specimens from 16 patients, together with 63 specimens from ICP were analysed by Southern hybridisation, using the mixed probe cocktail described above to screen for the presence of HPV DNA. As expected, detection of HPV DNA was greatest in VWs (64%), but 25% of keratoses, 24% of IECs and 33% of SCCs from RARs contained HPV DNA (Figure 2 and Table II). All specimens from normal skin were negative for HPV DNA. Lesions from ICPs showed lower HPV DNA prevalence than those from RARs, except for viral warts, only five of which were examined from ICPs (Table II). Statistical comparison of the results for each histological category between RARs, and ICPs revealed that SCC/RAR differed significantly (*P* < 0.05 by chi-squared test) from SCC/ICP, despite the small number of ICPs analysed. It should be noted that the two



**Figure 2** Southern hybridisation autoradiograph showing three HPV DNA-positive specimens, (A) SCC (from patient G), (D) VW and (F) VW, compared with three HPV DNA-negative specimens, (B) AK (from patient I who was HPV 16 positive by PCR), (C) IEC and (E) AK. All specimens were from RARs, and the size markers are indicated. A probe cocktail of HPV types 3, 8 and 13 was used with *Bam*HI-digested DNA. Track A (which was negative by PCR) shows evidence of HPV genome integration within a DNA fragment greater than 12 kb in size. Tracks D and F show episomal HPV genomes cleaved twice into fragments of 6 and 2 kb.



**Table II** HPV DNA prevalence detected by Southern hybridisation analysis

Patient group	VW	Number (%) of lesions positive				US
		K	IEC	SCC		
RARs	9/14 (64)	6/24 (25)	5/21 (24)	10/30 (33)	0/19 (0)	
ICPs	5/5 (100)	1/8 (13)	0/12 (0)	0/9 (0)	0/12 (0)	

Chi-squared tests revealed significant differences of  $P < 0.00001$  for comparisons of both VW/RAR with US/RAR and VW/ICP with US/ICP,  $P < 0.025$  for comparison of either K/RAR or IEC/RAR with US/RAR,  $P < 0.005$  for SCC/RAR vs US/RAR, and  $P < 0.05$  for SCC/RAR vs SCC/ICP.

RAR, renal allograft recipient; ICP, immunocompetent patient; VW, viral wart; K, keratosis; IEC, intraepidermal carcinoma; SCC, squamous cell carcinoma; US, uninvolved, sun-exposed skin.

groups of patients (RARs and ICPs) could not be age matched for IEC and SCC specimens, as these occurred mostly in the elderly in the ICP group. However, compared with 0% prevalence in US/RAR, HPV DNA positivities differed significantly in SCCs (33%;  $P < 0.005$ ), IECs (24%;  $P < 0.025$ ), Ks (25%;  $P < 0.025$ ) and VWs ( $P < 0.00001$ ) within the RAR group.

Restriction pattern analysis suggested that HPV integration had taken place in a dysplastic VW, an AK and an SCC (Figure 2) in three separate RARs. When digested with the single-cut enzyme *Bam*HI, both cases gave multiple restriction fragments, the sum of which was greater than 8 kb, but dissimilar to the size of multimer episomes. These banding patterns were reproducible, providing evidence that some RAR skin lesions contained integrated HPV DNA, but the numbers of affected lesions were too small to determine whether integration plays a significant role. The restriction patterns obtained when HPV-positive cases were further digested with *Pst*I were dissimilar, indicating that different HPV types were present in these lesions.

#### Detection of specific HPV types by polymerase chain reaction

The reaction conditions for all primers were optimised to allow detection of 0.001 pg of episomal HPV DNA in a background of 10 µg of placental DNA, equivalent to 80 copies of HPV or  $5 \times 10^{-5}$  copies per cell. Each set of primers was tested against a panel of cloned HPV types 1, 2, 3, 4, 5, 8, 10, 12, 14, 17, 19 and 20, and found to be absolutely type specific.

A total of 118 specimens from RARs and 48 from ICPs, were analysed by type-specific PCR for HPV types 1, 2, 5

and 8 (Tables III and IV). In each sample c-Ki-ras sequences could be detected with appropriate *ras* primers (data not shown). Relatively few specimens were positive for HPV DNA compared with results by Southern hybridisation analysis. In particular, HPV 5 DNA was only present in a small number of benign and premalignant lesions from RARs and ICPs but in no SCCs. HPV 8 DNA was found in only one SCC from an ICP. HPV 1 and 2 DNA was found in both benign and malignant lesions from RARs and ICPs (Tables III and IV). A total of 102 lesions from RARs and 43 from ICPs were further tested for the common genital HPV types 6, 11, 16 and 18 by type-specific PCR (Tables III and IV). 'High-risk' HPV 16 DNA was detected in uninvolved skin from an RAR, and 'low-risk' HPV 6 DNA was present in an SCC from an RAR. Four VWs from RARs contained more than one HPV type (5 and 2, 5 and 6, 5 and 11, 2 and 11). Rigorous anti-contamination procedures were followed throughout (Arends *et al.*, 1991), and there was no evidence to suggest that any of these positive results were due to contamination from other sources. Overall there was no dominant HPV type in any of the histological categories and the distribution of types was broadly similar for immunosuppressed and immunocompetent patients.

#### Correlation of HPV DNA detection by Southern hybridisation and type-specific PCR

Twenty-one specimens of Ks, IECs and SCCs from RARs exhibited HPV DNA by Southern hybridisation. However, only three of these were HPV DNA positive by type-specific PCR (Table V). Likewise, of the 13 specimens of Ks, IECs and SCCs from RARs that were HPV DNA positive by

**Table III** HPV type prevalence by type-specific PCR in renal allograft recipients

Histological type of lesion	Number examined	HPV type and number of positive lesions									
		1	2	5	8	Number examined	6	11	16	18	
VW	18	0	4	3	0	14	1	2	0	0	
K	26	2	1	1	0	23	0	0	1	0	
IEC	24	0	2	1	0	23	0	0	0	0	
SCC	31	1	2	0	0	24	1	0	1	0	
US	19	0	0	2	0	18	0	0	1	0	

VW, viral wart; K, keratosis; IEC, intraepidermal carcinoma; SCC, squamous cell carcinoma; US, uninvolved, sun-exposed skin.

**Table IV** HPV type prevalence by type-specific PCR in immunocompetent patients

Histological type of lesion	Number examined	HPV type and number of positive lesions									
		1	2	5	8	Number examined	6	11	16	18	
VW	6	1	1	0	0	5	0	0	0	0	
K	8	0	0	1	0	8	0	0	0	0	
IEC	13	2	1	1	0	11	0	0	0	0	
SCC	9	0	1	0	1	7	0	0	0	0	
US	12	0	1	0	0	12	0	0	0	0	

VW, viral wart; K, keratosis; IEC, intraepidermal carcinoma; SCC, squamous cell carcinoma; US, uninvolved, sun-exposed skin.

type-specific PCR, only three were positive by Southern hybridisation analysis (Table V). A combination of both detection assays resulted in 11/14 (79%) VWs, 10/24 (42%) Ks, 7/21 (33%) IECs, 13/30 (43%) SCCs and 3/19 (16%) USs from RARs containing HPV DNA (Table VI). The combined results for ICPs gave HPV prevalences of 5/5 (100%) for VWs, 2/8 (25%) for Ks, 3/13 (23%) for IECs, 2/9 (22%) for SCCs and 1/12 (8%) for USs. No statistically significant differences by the chi-squared test were found comparing HPV prevalence in each histological group between ICPs and RARs. However, SCCs from RARs showed a significantly higher HPV prevalence ( $P<0.05$ ) than uninvolved skin from RARs. Overall, HPV DNA was detected with greater frequency by Southern hybridisation analysis than by type-specific PCR (Tables II, III and IV). Some patients showed a high susceptibility to developing multiple malignant tumours exhibiting different HPV DNA content (Table V), but there was no specific pattern of combination of HPV types in these lesions.

Discussion

Prevalence of HPV DNA in the spectrum of cutaneous neoplasia in RAR

Compelling evidence exists of a contributory role for 'high-risk' genital HPV types 16 and 18 in the development of SCC of the genital tract (Arends *et al.*, 1990, 1991, 1993; zur Hausen, 1991; Lorincz *et al.*, 1992). Similarly, in EV the role of HPV 5 and 8 in the aetiopathogenesis of cutaneous SCC is suggested by their presence in over 90% of cancers (Orth *et al.*, 1979; Orth, 1986). Furthermore, HPV types 5, 8, 16 and 18 can cooperate with activated *ras* to transform rodent cells (Watts *et al.*, 1984; Iftner *et al.*, 1988; Fuchs & Pfister, 1990). By contrast, investigation of the relationship between HPV and cutaneous cancers in RARs has been inconclusive with regard to both prevalence and type of HPV DNA detected. This may be the result of differences in sample size studied or differences in sensitivity and specificity of the detection

Table V Clinicopathological details of HPV-positive lesions from renal allograft recipients

Code	Patient Age (years)/ sex (m/f)	Graft duration (years)	Histological type of lesion	Site	HPV type identified	
					Southern hybridisation	PCR
A	56m	6	AK (D++)	Face	pos uk	neg
B	49m	17	SCC	Scalp	pos	2
B			SCC	Ear	pos uk	neg
C	57m	12	AK (D++)	Finger	pos uk	neg
C			SCC	Hand	pos uk	neg
C			IEC	Ear	pos uk	neg
D	55m	8 (cardiac)	AK (D++)	Dorsum hand	pos uk	neg
D			VK	Dorsum hand	pos 10 <sup>a</sup>	neg
E	36f	21	IEC	Presternal	pos	5
E			VK	Thigh	ND	5
E			SCC	Neck	neg	16
E			SCC	Chest	neg	2
E			SCC	Chest	pos uk	neg
E			IEC	Dorsum hand	pos uk	neg
E			SCC	Forearm	neg	6
F	52m	10	IEC	Upper back	pos uk	neg
F			SCC	Neck	pos uk	neg
F			SCC	Back	pos uk	neg
F			VW	Dorsum hand	neg	5,11
F			SCC	Shoulder	pos	1
G	44m	26	IEC	Scalp	neg	2
G			SCC	Forearm	pos uk	neg
G			SCC	Scalp	pos uk	neg
G			IEC	Scalp	pos uk	neg
G			SCC	Chest	pos uk	neg
H	59m	13	IEC	Scalp	neg	2
I	62m	8	AK (D++)	Forearm	neg	16
I			VK	Forearm	pos uk	neg
J	52m	15	VK (D++)	Forearm	neg	1
J			AK	Forearm	neg	1
J			VK	Forearm	pos uk	neg
J			VK (D+)	Thigh	neg	2

AK, actinic keratosis; D+, mild dysplasia; D++, moderate dysplasia; D+++, severe dysplasia; SCC, squamous cell carcinoma; IEC, intraepidermal carcinoma; VK, verrucous keratosis; ND, not done; uk, unknown; neg, negative. <sup>a</sup>The *PstI* and *HindIII* restriction digest of this lesion gave identical restriction fragment patterns to HPV 10 by Southern hybridisation analysis.

Table VI Combined HPV prevalence by Southern hybridisation analysis and type-specific PCR assays

Patient group	VW	Number (%) lesions positive			
		K	IEC	SCC	US
RARs	11/14 (79)	10/24 (42)	7/21 (33)	13/30 (43)	3/19 (16)
ICPs	5/5 (100)	2/8 (25)	3/13 (23)	2/9 (22)	1/12 (8)

Chi-squared tests revealed significant difference of  $P<0.05$  for comparisons of SCC/RAR with US/RAR, and  $P=0.00003$  for both VW/RAR vs US/RAR and VW/ICP vs US/ICP.

methods employed (Lutzner *et al.*, 1980, 1983; Rudlinger *et al.*, 1986; Jablonska *et al.*, 1987; Van der Leest, 1987; Barr *et al.*, 1989; Blessing *et al.*, 1989; Rudlinger & Grob, 1989; Euvrard *et al.*, 1991). An additional factor in some studies may be the inclusion of a large proportion of patients who seem to be at exceptionally high risk of developing multiple and widespread premalignant and malignant cutaneous lesions with increased HPV DNA content (Barr *et al.*, 1989). To overcome some of these problems we have studied a large, unselected series of RARs using both Southern hybridisation and type-specific PCR techniques.

The prevalence of HPV DNA was closely similar throughout the spectrum of cutaneous neoplasia in RARs: 42% of keratoses, 33% of IECs and 43% of SCCs contained HPV DNA, but only 16% of uninvolved skin was positive (Table VI), not dissimilar to some other reports (Soler *et al.*, 1992). This pattern differs from that found in cervical neoplasia in which the prevalence of HPV (of specific 'high-risk' types) increases throughout the cervical intraepithelial neoplasia spectrum (Stanley, 1990; Arends *et al.*, 1991, 1993; Lorincz *et al.*, 1992). These findings suggest that, if HPV play a role in cutaneous neoplasia of RARs, this must involve the early stages of the neoplastic process. One hypothesis is that HPVs may act as tumour promoters by stimulating cell proliferation. Thus, HPV may provide a stimulus analogous to that of phorbol esters in traditional rodent skin carcinogenesis models, in which promotion of cell proliferation fixes irreversibly any genetic mutations induced by initiating agents such as chemical carcinogens or UV light. The role of promoter for HPV was previously suggested by zur Hausen (1982), and the ability to induce keratinocyte proliferation is common to the many different types of common cutaneous and EV-associated HPV, as evidenced by the variety of warts that they cause. This hypothesis is supported by the clinicopathological observations that RARs frequently exhibit extensive warts and verrucous keratoses, as well as malignant tumours: these lesions form a seamless spectrum of histological change, with many keratoses (actinic and verrucous) showing dysplasia, and both IECs and SCCs retaining viral features. Furthermore, these immunosuppressed patients appear to have relatively high background levels of HPV, indicated by our findings that 3 out of 19 (16%) biopsies from the apparently uninvolved skin of RARs contained HPV DNA, in two cases HPV 5 DNA and in one HPV 16 DNA. Such a background level of HPV infection of skin, in the presence of long-term immunosuppression that is likely to permit viral persistence over a prolonged period, together with sun-induced DNA damage, may give rise to conditions conducive to tumour induction. Moreover, the usual 1:5 ratio of SCC-BCC is reversed in RARs to 15:1, suggesting that HPVs encourage squamous rather than basal cell neoplastic differentiation.

#### *Specific HPV types found in cutaneous neoplasms in RAR*

In this study we were not always able to characterise fully the HPV types found by Southern hybridisation analysis. However, the results of type-specific PCR analysis indicate that only a small proportion of lesions contained HPV 5 or 8 DNA. This is in contrast to previous findings from SE Scotland (Barr *et al.*, 1989), where 15 out of 25 SCCs were

found to contain HPV5 or 8 DNA. That study used mostly dot blotting to detect HPV 5 or 8 DNA, which does not exclude the possibility of cross-hybridisation with other EV-associated HPV types. Moreover, all 15 positive specimens in that study came from four patients at exceptionally high risk of development of cutaneous lesions, each of whom had multiple SCCs. The balance of evidence now suggests that HPV 5 and 8 DNA is found relatively infrequently in tumours from RARs (Lutzner *et al.*, 1980, 1983; Rudlinger *et al.*, 1986; Van der Leest, 1987; Soler *et al.*, 1992).

It is of interest that we found both HPV 1 and 2 DNA by PCR in a small number of SCCs in this series. These HPV types were previously considered to be non-transforming, and usually associated with benign skin warts. Recent work has also emphasised the importance of extending investigations of HPV content to include the anogenital HPV types (Ostrow *et al.*, 1987, 1989; Stone *et al.*, 1987; Rudlinger *et al.*, 1989; Eliezri *et al.*, 1990; Ashinoff *et al.*, 1991). Two positive SCCs in the present study, one containing HPV 6 and the other HPV 16 DNA, both came from a female RAR who in addition to multiple cutaneous SCCs has developed SCCs of cervix, vulva and anal canal. HPV 16 DNA has also been detected in her genital tumours. Overall, from the present investigation using both Southern hybridisation and PCR, the emerging pattern of HPV type prevalence is one of involvement by multiple HPV types.

Some cases found to contain HPV sequences by PCR could not be confirmed by Southern hybridisation analysis, indicating that in many cases copy numbers of HPV genomes were too low to be detected by Southern analysis. Not surprisingly, the absolutely type-specific PCR assays for HPV 1, 2, 5, 6, 8, 11, 16 and 18 did not detect other HPV types found by Southern hybridisation. In a pilot study applying a consensus PCR assay (Manos *et al.*, 1989), primarily designed to detect genital HPV types, the common cutaneous and EV-associated HPV types were poorly detected even when using cloned HPV plasmid DNA as template (unpublished data). Thus, it is possible that this and other studies have underestimated the true HPV prevalence in cutaneous neoplasms in RARs, owing to a combination of a wide variety of HPV types involved and low copy number of HPV genomes.

The overall pattern found in this study is of similar HPV prevalence throughout the spectrum of cutaneous neoplasia in RARs. Furthermore, studies of the prevalence of accurately typed specific HPV have shown that no single HPV type predominates in cutaneous lesions in RARs with multiple HPV types being detected. At a practical level, these data challenge the necessity to systematically type HPV DNA found in cutaneous lesions in RARs. Our observations are consistent with the hypothesis that in RARs multiple HPV types play a role in carcinogenesis by promotion of cell proliferation, and this hypothesis merits further testing.

We would like to thank the Scottish Home and Health Department and Cancer Research Campaign for funding this research, Robert Morris for technical advice, Jill Bubb and Andrew Wyllie for useful discussions and Miss Jenni Westwater for secretarial assistance.

#### References

- ALLOUB, M.I., BARR, B.B.B., MCLAREN, K.M., SMITH, I.W., BUNNEY, M.H. & SMART, G.E. (1989). Human papillomavirus and lower genital neoplasia in renal transplant patients. *Obstet. Gynecol.*, **68**, 251–258.
- ARENDS, M.J., WYLLIE, A.H. & BIRD, C.C. (1990). Papillomaviruses and human cancer. *Hum. Pathol.*, **21**, 686–698.
- ARENDS, M.J., DONALDSON, Y.K., DUVAL, E., WYLLIE, A.H. & BIRD, C.C. (1991). HPV in full thickness cervical biopsies: high prevalence in CIN 2 and CIN 3 detected by a sensitive PCR method. *J. Pathol.*, **165**, 301–309.
- ARENDS, M.J., DONALDSON, Y.K., DUVAL, E., WYLLIE, A.H. & BIRD, C.C. (1993). Human papillomavirus type 18 associates with more advanced cervical neoplasia than human papillomavirus type 16. *Hum. Pathol.*, **24**, 432–437.
- ASHINOFF, R., LI, J.J., JACOBSON, M., FRIEDMAN-KEIN, A.E. & GERONEMUS, R.G. (1991). Detection of human papillomavirus DNA in squamous cell carcinoma of the nail bed and finger determined by polymerase chain reaction. *Arch. Dermatol.*, **127**, 1813–1818.



- BAADSGAARD, O. (1991). *In vivo* ultraviolet irradiation of human skin results in profound perturbation of the immune system. *Arch. Dermatol.*, **127**, 99–109.
- BARR, B.B., BENTON, E.C., MCLAREN, K.M., BUNNEY, M.H., SMITH, I.W., BLESSING, K. & HUNTER, J.A.A. (1989). Human papilloma virus infection and skin cancer in renal allograft recipients. *Lancet*, **i**, 124–129.
- BENTON, C., SHAHIDULLAH, H. & HUNTER, J.A.A. (1992). Human papillomavirus in the immunosuppressed. *Papillomavirus Rep.*, **3**, 23–26.
- BIRKELAND, S.A. (1983). Malignant tumours in renal transplant patients. *Cancer*, **51**, 1571–1575.
- BLESSING, K., MCLAREN, K.M., BENTON, E.C., BARR, B.B., BUNNEY, M.H., SMITH, I.W. & BEVERIDGE, G.W. (1989). Histopathology of skin lesions in renal allograft recipients – an assessment of viral features and dysplasia. *Histopathology*, **14**, 129–139.
- BLOHME, I. & LARKO, O. (1984). Premalignant and malignant skin lesions in renal transplant patients. *Transplantation*, **37**, 165–167.
- BOYLE, J., MACKIE, R.M., BRIGGS, J.D., JUNOR, B.J.R. & AITCHISON, T.C. (1984). Cancer, warts and sunshine in renal transplant patients. A case control study. *Lancet*, **i**, 702–705.
- DANOS, O., KATINKA, M. & YANIV, M. (1982). Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among Papovaviridae. *EMBO J.*, **1**, 231–236.
- DYALL-SMITH, D., TROWELL, H., MARK, A. & DYALL-SMITH, M. (1991). Cutaneous squamous cell carcinomas and papillomaviruses in renal transplant recipients: a clinical and molecular biological study. *J. Dermatol. Sci.*, **2**, 139–146.
- ELIEZRI, Y.D., SILVERSTEIN, S.J. & NUOVO, G.J. (1990). Occurrence of human papillomavirus type 16 DNA in cutaneous squamous and basal cell neoplasms. *J. Am. Acad. Dermatol.*, **23**, 836–842.
- EUVRARD, S., CHARDONNET, Y., DUREAU, G., HERMIER, C. & THIVOLET, J. (1991). Human papillomavirus type 1-associated squamous cell carcinoma in a heart transplant recipient. *Arch. Dermatol.*, **127**, 559–564.
- FUCHS, P.G. & PFISTER, H. (1990). Papillomaviruses in epidermodysplasia verruciformis. *Papillomavirus Rep.*, **1**, 1–4.
- FUCHS, P.G., IFTNER, T., WENINGER, J. & PFISTER, H. (1986). Epidermodysplasia verruciformis-associated human papillomavirus 8: genomic sequence and comparative analysis. *J. Virol.*, **58**, 626–634.
- GASSENMAIER, A., LANNEL, M. & PFISTER, H. (1984). Molecular cloning and characterization of the DNAs of human papillomavirus 19, 20 and 25 from a patient epidermodysplasia verruciformis. *J. Virol.*, **52**, 1019–1023.
- HEILMAN, L.A., LAW, M.F., ISRAEL, M.A. & HOWLEY, P.M. (1980). Cloning of human papilloma virus genomic DNAs and analysis of homologous polynucleotide sequences. *J. Virol.*, **36**, 395–407.
- HIRSCH-BEHNAM, A., DELIUS, H. & DE VILLIERS, E.M. (1990). A comparative sequence analysis of two human papillomavirus (HPV) types 2a and 57. *Virus Res.*, **18**, 81–98.
- HOLGATE, C.S., JACKSON, P., POLLARD, K., LUNNY, D. & BIRD, C.C. (1986). Effect of fixation on T and B lymphocyte surface membrane antigen demonstration in paraffin processed tissue. *J. Pathol.*, **149**, 293–300.
- HOXTTELL, U.E., MANDEL, J.S., MURRAY, S.S., SCHUMAN, L.M. & GOLTZ, R.W. (1977). Incidence of skin carcinoma after renal transplantation. *Arch. Dermatol.*, **113**, 437–438.
- IFTNER, T., BIERFELDER, S., CSAPO, Z. & PFISTER, H. (1988). Involvement of human papillomavirus type 8 genes E6 and E7 in transformation and replication. *J. Virol.*, **62**, 3655–3661.
- JABLONSKA, S., KAWASHIMA, M., OBALEK, S., SZYMANCZYK, J. & ORTH, G. (1987). Human papillomavirus-related cutaneous benign lesions and skin malignancies. *Cancer Cells*, **5**, 309–317.
- KREMSDORF, D., JABLONSKA, S., FAVRE, M. & ORTH, G. (1983). Human papillomaviruses associated with epidermodysplasia verruciformis. II. Molecular cloning and biochemical characterization of human papillomavirus 3a, 8, 10 and 12 genomes. *J. Virol.*, **48**, 340–351.
- KREMSDORF, D., FAVRE, M., JABLONSKA, S., OBALEK, S., RUEDA, L.A., LUTZNER, M.A., BLANCHET-BARDON, C., VADER, P.C.V.V. & ORTH, G. (1984). Molecular cloning and characterization of the genomes of nine newly recognized human papillomavirus types associated with epidermodysplasia verruciformis. *J. Virol.*, **52**, 1013–1018.
- LORINCZ, A.T., REID, R., JENSON, A.B., GREENBERG, M.D., LANCASTER, W.D. & KURMAN, R.J. (1992). Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. *Obstet. Gynecol.*, **79**, 328–337.
- LUTZNER, M.A., ORTH, G. & DUTRONQUAY, V. (1983). Detection of human papillomavirus type 5 DNA in skin cancers of an immunosuppressed renal allograft recipient. *Lancet*, **i**, 422–424.
- LUTZNER, M., CROISSANT, O., DUCASSE, M.F., KREIS, H., CROSNIER, J. & ORTH, G. (1980). A potentially oncogenic human papillomavirus (HPV-5) found in two renal allograft recipients. *J. Invest. Dermatol.*, **75**, 353–356.
- MANOS, M., TING, M.Y., WRIGHT, D.K., LEWIS, A.J., BROKER, T.R. & WOLINSKY, S.M. (1989). The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells*, **7**, 209–214.
- ORTH, G. (1986). Epidermodysplasia verruciformis. In *The Papoviridae*, Vol. 2. *The Papillomaviruses*. Salzman, N.P. & Howley, P.M. (eds). Plenum: New York.
- ORTH, G., JABLONSKA, S., JARZABEK-CHORZELSKA, M., OBALEK, S., RZESA, G., FAVRE, M. & CROISSANT, O. (1979). Characteristics of the lesions at risk of malignant conversion associated with the type of human papillomavirus involved in epidermodysplasia verruciformis. *Cancer Res.*, **39**, 1074–1082.
- OSTROW, R.S., BENDER, M., NIMURA, M., SEKI, T., KAWASHIMA, M., PASS, F. & FARAS, A.J. (1982). Human papillomavirus DNA in cutaneous primary and metastasized squamous cell carcinomas from patients with epidermodysplasia verruciformis. *Proc. Natl Acad. Sci. USA*, **79**, 1634–1638.
- OSTROW, R., ZACHOW, K., WATTS, S., BENDER, M., PASS, F. & FARAS, A. (1983). Characterization of two HPV 3 related papillomaviruses from common warts that are distinct from flat warts or epidermodysplasia verruciformis. *J. Invest. Dermatol.*, **80**, 436–440.
- OSTROW, R., MANIAS, D. & MITCHELL, A. (1987). Epidermodysplasia verruciformis: a case associated with primary lymphatic dysplasia, depressed cell-mediated immunity, and Bowen's disease containing human papillomavirus 16 DNA. *Arch. Dermatol.*, **123**, 1511–1516.
- OSTROW, R.S., SHAVER, K., TURNQUIST, S., VIKSNINS, A., BENDER, M., VANCE, C., KAYE, V. & FARAS, A.J. (1989). Human papillomavirus-16 DNA in a cutaneous invasive cancer. *Arch. Dermatol.*, **125**, 666–669.
- PFISTER, H., GASSENMAIER, A., NURNBERGER, F. & STUTTGEN, G. (1983a). Human papilloma virus 5-DNA in a carcinoma of an epidermodysplasia verruciformis patient infected with various human papillomavirus types. *Cancer Res.*, **43**, 1436–1441.
- PFISTER, H., HETTICH, I., RUNNE, U., GISSMANN, L. & CHILF, G.N. (1983b). Characterization of human papillomavirus type 13 from focal epithelial hyperplasia Heck lesions. *J. Virol.*, **47**, 363–366.
- RUDLINGER, R. & GROB, R. (1989). Papillomavirus infection and skin cancer in renal allograft recipients. *Lancet*, **i**, 1132–1133.
- RUDLINGER, R., SMITH, I.W., BUNNEY, M.H. & HUNTER, J.A.A. (1986). Human papillomavirus infections in a group of renal transplant recipients. *Br. J. Dermatol.*, **115**, 681–692.
- RUDLINGER, R., GROB, R., YU, Y.X. & SCHNYDER, U.W. (1989). Human papillomavirus-35-positive Bowenoid papulosis of the anogenital area and concurrent human papillomavirus-35-positive verruca with Bowenoid dysplasia of the periungual area. *Arch. Dermatol.*, **125**, 655–659.
- SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. (1989). *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.
- SHEIL, A.G.R., FLAVEL, S., DISNEY, A.P.S. & MATHEW, T.H. (1985). Cancer development in patients progressing to dialysis and renal transplantation. *Transplantation Proc.*, **17**, 1685–1692.
- SHUTTLEWORTH, D., MARKS, R., GRIFFIN, P.J.A., SALAMAN, J.R. (1987). Dysplastic epidermal change in immunosuppressed patients with renal transplants. *Q. J. Med.*, **243**, 609–616.
- SOLER, C., CHARDONNET, Y., EUVRARD, S., CHIGNOL, M.C. & THIVOLET, J. (1992). Evaluation of human papillomavirus type 5 on frozen sections of multiple lesions from transplant recipients with *in situ* hybridisation and non-isotopic probes. *Dermatology*, **184**, 248–253.
- STANLEY, M. (1990). Genital papillomaviruses, polymerase chain reaction and cervical cancer. *Genitourin. Med.*, **66**, 415–417.
- STONE, M., NOONAN, C. & TSCHEN, J. (1987). Bowen's disease of the feet: presence of human papillomavirus 16 DNA in tumour tissue. *Arch. Dermatol.*, **123**, 1517–1520.

- STREILEIN, J.W. (1991). Immunogenetic factors in skin cancer. *N. Engl. J. Med.*, **325**, 885-886.
- VAN DER LEEST, R.J., ZACHOW, K.R., OSTROW, R.S., BENDER, M., PASS, F. & FARAS, A.J. (1987). Human papillomavirus heterogeneity in 36 renal transplant recipients. *Arch. Dermatol.*, **123**, 354-357.
- WATTS, S.L., PHELPS, W.C., OSTROW, R.S., ZACHOW, K.R. & FARAS, A.J. (1984). Cellular transformation by human papillomavirus DNA *in vitro*. *Science*, **225**, 634-636.
- YABE, Y., TANIMURA, Y., SAKIA, A., HITSUMOTO, T. & NOHARA, N. (1989). Molecular characteristics and physical state of human papillomavirus DNA change with progressing malignancy: studies in a patient with epidermodysplasia verucciformis. *Int. J. Cancer*, **43**, 1022-1028.
- ZACHOW, K.R., OSTROW, R.S. & FARAS, A.J. (1987). Nucleotide sequence and genome organization of human papillomavirus type 5. *Virology*, **158**, 251-254.
- ZUR HAUSEN, H. (1982). Human genital cancer: synergism between two virus infections or synergism between a virus infection and initiating events. *Lancet*, **ii**, 1370-1372.
- ZUR HAUSEN, H. (1991). Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology*, **184**, 9-13.



## Accumulation of p53 is associated with tumour progression in cutaneous lesions of renal allograft recipients

L.A. Stark<sup>1</sup>, M.J. Arends<sup>1</sup>, K.M. McLaren<sup>1</sup>, E.C. Benton<sup>2</sup>, H. Shahidullah<sup>2</sup>, J.A.A. Hunter<sup>2</sup> & C.C. Bird<sup>1</sup>

<sup>1</sup>Department of Pathology, Edinburgh University Medical School, Teviot Place, Edinburgh, UK; <sup>2</sup>Department of Dermatology, Royal Infirmary of Edinburgh, Lauriston Place, Edinburgh, UK.

**Summary** Renal allograft recipients suffer from a markedly increased susceptibility to premalignant and malignant cutaneous lesions. Although various aetiological factors have been implicated, little is known of the associated genetic events. In this study we initially employed immunocytochemical techniques to investigate the prevalence and localisation of accumulated p53 in over 200 cutaneous biopsies (including 56 squamous cell carcinomas) from renal allograft recipients and immunocompetent controls. In renal allograft recipients accumulated p53 was present in 24% of uninvolved skin samples, 14% of viral warts, 41% of premalignant keratoses, 65% of intraepidermal carcinomas and 56% of squamous cell carcinomas [squamous cell carcinoma and intraepidermal carcinoma differed significantly from uninvolved skin ( $P < 0.005$ ) and viral warts ( $P < 0.01$ )]. A similar trend was revealed in immunocompetent patients (an older, chronically sun-exposed population) but with lower prevalence of p53 immunoreactivity: 25% of uninvolved skin samples, 0% of viral warts, 25% of keratoses, 53% of intraepidermal carcinomas and 53% of squamous cell carcinomas. These differences were not statistically significant. Morphologically, p53 immunoreactivity strongly associated with areas of epidermal dysplasia and the abundance of staining correlated positively with the severity of dysplasia. These data suggest that p53 plays a role in skin carcinogenesis and is associated with progression towards the invasive state. No correlation was observed between accumulated p53 and the presence of human papillomavirus (HPV) DNA in any of the lesions. Single-strand conformational polymorphism analysis (exons 5–8) was used to determine the frequency of mutated p53 in 28 malignancies with varying degrees of immunopositivity. p53 mutations were found in 5/9 (56%) malignancies with p53 staining in  $> 50\%$  of cells, reducing to 1/6 (17%) where 10–50% of cells were positively stained and none where  $< 10\%$  of cells were stained. These data imply that factors other than p53 gene mutation play a part in accumulation of p53 in skin cancers.

The p53 gene encodes a 53 kDa phosphoprotein that acts as a transcription factor and has tumour-suppressor functions. The wild-type gene product also has the ability to induce growth arrest and/or apoptosis in response to DNA injury, preventing replication of genomes that have suffered DNA damage (Kastan *et al.*, 1991; Hartwell, 1992; Lane, 1992, 1993; Unger *et al.*, 1992; Clark *et al.*, 1993; Hall *et al.*, 1993). Mutations in the p53 gene are considered to play a significant part in the development of many human malignancies: a high frequency of mutation is observed in most of the common forms of human cancer and there are elevated rates of malignancy in patients with Li–Fraumeni syndrome (in which there is an inherited p53 gene mutation) and in genetically engineered, p53-deficient mice (Baker *et al.*, 1989; Nigro *et al.*, 1989; Srivastava *et al.*, 1990; Hollstein *et al.*, 1991; Donehower *et al.*, 1992; Purdie *et al.*, 1994). A number of oncogenic viral proteins can also form complexes with wild-type p53, initiating gene inactivation by mechanisms other than mutational loss of function (Scheffner *et al.*, 1990; Yew & Berk, 1992; Debbas & White, 1993; Moran, 1993). Most of the mutations observed in p53 are thought to induce conformational changes in the protein product, increasing its half-life and rendering it detectable by immunocytochemical techniques (Milner & Cook, 1986; Gannon *et al.*, 1990; Milner & Medcalf, 1991; Montenarh, 1992; Wynford-Thomas, 1992).

Renal allograft recipients (RARs) manifest a greatly increased susceptibility to cutaneous malignancy, with squamous cell carcinoma (SCC) occurring commonly, especially in patients with long graft life or high sun exposure (Shuttleworth *et al.*, 1987; Alloub *et al.*, 1989; Benton *et al.*, 1992). These malignancies, however, form part of the wider spectrum of cutaneous disease observed in RARs that includes viral warts (VWs) and keratoses (Ks) displaying varying

degrees of epidermal dysplasia and topographical continuity with intraepidermal carcinoma (IEC) and invasive SCC (Blessing *et al.*, 1989; Benton *et al.*, 1992). Although a number of putative aetiological factors have been implicated in the development of these malignancies, including ultraviolet (UV) radiation (Blohme & Larko, 1984; Boyle *et al.*, 1984), decreased cell-mediated immunity (Streilein, 1991) and human papillomavirus (HPV) infection (Rudlinger *et al.*, 1986; Barr *et al.*, 1989; Benton *et al.*, 1992; Stark *et al.*, 1994), little is known of the associated genetic events and whether these may differ in RARs and immunocompetent patients (ICPs). To our knowledge, there have been no major studies in which the role of p53 in the development of cutaneous lesions in RARs has been considered, although p53 mutations have been reported to occur in IECs and SCCs from ICPs (Brash *et al.*, 1991; Gusterson *et al.*, 1991; Pierceall *et al.*, 1991; McGregor *et al.*, 1992; Burns *et al.*, 1993; Campbell *et al.*, 1993a, b).

In this study we have employed immunocytochemical techniques to compare the prevalence of p53 accumulation in premalignant and malignant cutaneous lesions from both RARs and ICPs. Single-strand conformational polymorphism (SSCP) analysis was also employed to determine the relationship between positive immunocytochemistry and p53 gene mutations. The relationship between p53 expression and the HPV status of the lesions was also considered since it has been reported that viral oncoproteins may play a part in p53 inactivation in other HPV-associated malignancies (Scheffner *et al.*, 1990, 1991, 1992; Werness *et al.*, 1990; Crook *et al.*, 1991, 1992).

### Materials and methods

#### Patients

Sixty RARs (mean age 49 years, range 20–71 years) and 83 ICPs (mean age 68 years, range 12–94 years) were investigated. All SCCs came from 10 RARs and 17 ICPs. RARs

received transplants between 1965 and 1992 (mean duration of transplant 10.8 years, range 1–26 years). Prior to 1984, prednisolone and azathioprine were the main immunosuppressive drugs used, but thereafter most patients received prednisolone and cyclosporin A. ICPs all presented to the Dermatology Department in Edinburgh Royal Infirmary for treatment of viral warts or skin tumours. Most of these patients were elderly with lesions on sun-exposed sites.

#### Tissue collection

One hundred and thirty-five and 68 cutaneous lesions were collected from RARs and ICPs respectively. These included 56 SCCs and 62 IECs. Six millimetre punch biopsies of normal (sun-exposed), forearm skin were also collected from 21 RARs and 12 ICPs. Biopsy samples were bisected longitudinally; half were placed immediately in PLPD (periodate-lysine-paraformaldehyde-dichromate) (Holgate *et al.*, 1986), or 10% formalin and fixed for 24 h at 4°C before paraffin embedding. Histological assessment and immunohistochemistry were carried out on sections prepared from paraffin-embedded material. The other half were snap frozen in liquid nitrogen and stored at -70°C to await DNA extraction and virological investigation.

#### DNA extraction and HPV detection

Frozen tissue was minced in lysis buffer (50 mM Tris, 50 mM EDTA, 100 mM sodium chloride, 5 mM DTT, 1% SDS 1.5 mg ml<sup>-1</sup> proteinase K) then incubated at 37°C overnight. DNA extraction was carried out using a standard phenol-chloroform extraction technique (Sambrook *et al.*, 1989). Two methods were employed to screen for the presence of HPV DNA (Stark *et al.*, 1994). Southern analysis, using mixed HPV probes at low hybridisation ( $T_m$  - 40°C) and washing stringency ( $T_m$  - 35°C), was used to detect common cutaneous and epidermodysplasia verruciformis (EV)-related types. The polymerase chain reaction (PCR) was used to detect specific HPV types 1, 2, 5, 8, 6, 11, 16 and 18 (Arends *et al.*, 1991; Stark *et al.*, 1994).

#### Histopathology

The skin lesions were classified as follows: viral warts (VWs) exhibited symmetry, papilliferous architecture and koilocytic change; verrucous keratoses (VKs) displayed the architecture of warts but lacked definitive cytological features of viral infection; actinic keratoses (AKs) showed basal budding and basal hypermelanosis (degrees of dysplasia were assessed in both types of keratosis); intraepidermal carcinoma (IECs) showed either full-thickness dysplasia or severe dysplasia and acantholysis of the basal layer, invasive squamous cell carcinoma (SCC) showed dermal invasion (Blessing *et al.*, 1989).

#### Immunocytochemistry

Immunocytochemistry was performed on 3 µm sections of PLPD- and formalin-fixed tissue using the mouse anti-p53 monoclonal antibodies MAb Do-7 (Vojtesek *et al.*, 1992) and PAb 1801 (Banks *et al.*, 1986) and a standard ABC horseradish peroxidase (HRP) technique (Dako, High Wycombe, Bucks, UK) as previously described (Purdie *et al.*, 1991). Formalin-fixed tissue was treated with MAb Do-7 (1:100 dilution, overnight incubation) only, whereas PLPD-fixed material was treated with MAb Do-7 and PAb 1801 (1:100 dilution, 1 h incubation). Each section was scored by two independent observers and the extent of staining recorded on the following graded scale: 1 = <10%, 2 = 10–50% and 3 = >50% of cells in a lesion showing positive nuclear staining. Sections were recorded as positive when immune precipitate was visible in >10% of cells in the lesion, i.e. grades 2 and 3 only. Lesions with grade 1 score were considered to be negative. The histological localisation of accumulated p53 within each lesion was also noted.

#### Single-strand conformational polymorphism (SSCP) analysis and direct DNA sequencing

Twenty-eight tumour samples and 12 normal skin samples from RARs and ICPs underwent SSCP analysis. PCR was performed on 0.1–1 µg of genomic DNA using primers specific for p53 exons 5, 6, 7 and 8. SSCP analysis was based on the protocol of Cripps *et al.* (manuscript in preparation). The 100 µl PCR reaction was purified using a standard chloroform extraction technique. A 5–10 µl volume of the purified product was alkali denatured (80 µM sodium hydroxide, 10 µM EDTA, at 48°C for 5 min), 10 µl of stop solution added (10 mM EDTA, 0.1% bromophenol blue, 0.01% xylene cyanol) and the whole sample loaded onto a 5% glycerol, 0.5 × MDE Hydrolink gel. Following electrophoresis (25°C, 20 W, for 2–3 h) the DNA was visualised by silver staining (BioRad kit). SSCP mutations were detected as bands of altered mobility. In one sample showing an exon 7 mutation by SSCP analysis, sequencing was performed using the Sequenase (II) kit (United States Biochemical) with cloned double-stranded DNA.

#### Results

##### Immunocytochemical demonstration of p53

Experiments were initially carried out to determine the specificity and sensitivity of MAb Do-7 and PAb 1801 staining in PLPD- and formalin-fixed material. No statistically significant difference in the number of positive cases was detected in formalin- or PLPD-fixed material (data not shown), permitting results from both fixatives to be combined. In 74 lesions tested with both MAb Do-7 and PAb 1801 the number of positive cases was identical, and within each section both antibodies reacted with similarly located cells. Overall, MAb Do-7 gave a more intense precipitate than PAb 1801, although some minor variation in intensity occurred between assays.

##### Accumulated p53 in cutaneous lesions from RARs and ICPs

A total of 156 biopsies from RARs and 80 from ICPs were screened for the presence of accumulated p53 using MAb Do-7 (Table I, Figures 1 and 2). In both populations, over 50% of SCCs exhibited p53 immunoreactivity in >10% of cells (grades 2 and 3). Overall, the number of lesions exhibiting accumulated p53 and the grade of staining within these lesions correlated positively with the degree of dysplasia present. In RARs, significantly more IECs and SCCs demonstrated accumulated p53 than either uninvolved sun-exposed skin (US) ( $\chi^2$  test,  $P < 0.05$ ) or VWs ( $\chi^2$  test,  $P < 0.01$ ). A similar trend was revealed in ICPs, although a lower proportion of cases were stained positive for p53. However, the differences between SCCs or IECs and US in ICP were not statistically significant.

##### Distribution of accumulated p53

In both RARs and ICPs, immunostaining of lesions was confined to nuclei of dysplastic epithelial cells and was most

**Table I** Prevalence of accumulated p53 in cutaneous lesions from RAR and ICPs

Patients	No. of lesions (%) demonstrating accumulated p53 <sup>a</sup>				
	US	VWs	Ks	IECs	SCCs
RARs	5/21 (24) <sup>b</sup>	3/21 (14) <sup>c</sup>	17/41 (41)	22/34 (65) <sup>b,c</sup>	22/39 (56) <sup>b,c</sup>
ICPs	3/12 (25)	0/7 (0)	4/16 (25)	15/28 (53)	9/17 (53)

<sup>a</sup>Sections with staining in >10% of nuclei in the lesion (grades 2 and 3) were scored as positive. RAR, renal allograft recipient; ICP, immunocompetent patient; VW, viral wart; K, verrucous and actinic keratosis; IEC, intraepidermal carcinoma; SCC, squamous cell carcinoma; US, uninvolved, sun-exposed skin. <sup>b</sup> $P < 0.05$  using  $\chi^2$  test. <sup>c</sup> $P < 0.01$  using  $\chi^2$  test.

abundant in areas of severe dysplasia (Figure 2a). Within K and IEC lesions, staining was generally strongest in basal epithelial layers, particularly at sites of basal budding where dysplastic changes were most severe (Figure 2a and b). This was particularly notable in Ks exhibiting actinic features. In dysplastic Ks and IECs, acantholysis and suprabasal clefting were also observed to correlate with strong p53 staining. In tissue sections that contained skin appendages, the specialised lining cells were always negative and staining was confined to the surrounding dysplastic cells (Figure 2c). While the majority of SCCs showed accumulated p53, there was a tendency for greater positivity to occur in less well-differentiated lesions (Figure 2d) and adjacent normal epidermis remained unstained. Occasionally, p53 was detected in dysplastic basal cells and overlying IECs but not in contiguous tongues of invasive carcinoma. The positive staining in non-lesional, sun-exposed skin was light in intensity and

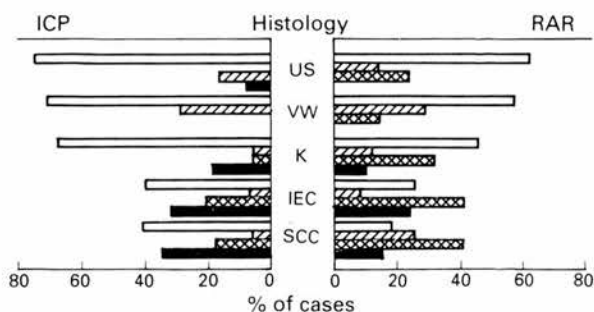
predominantly basal in location in cells exhibiting only mild dysplastic change.

#### HPV status and presence of accumulated p53

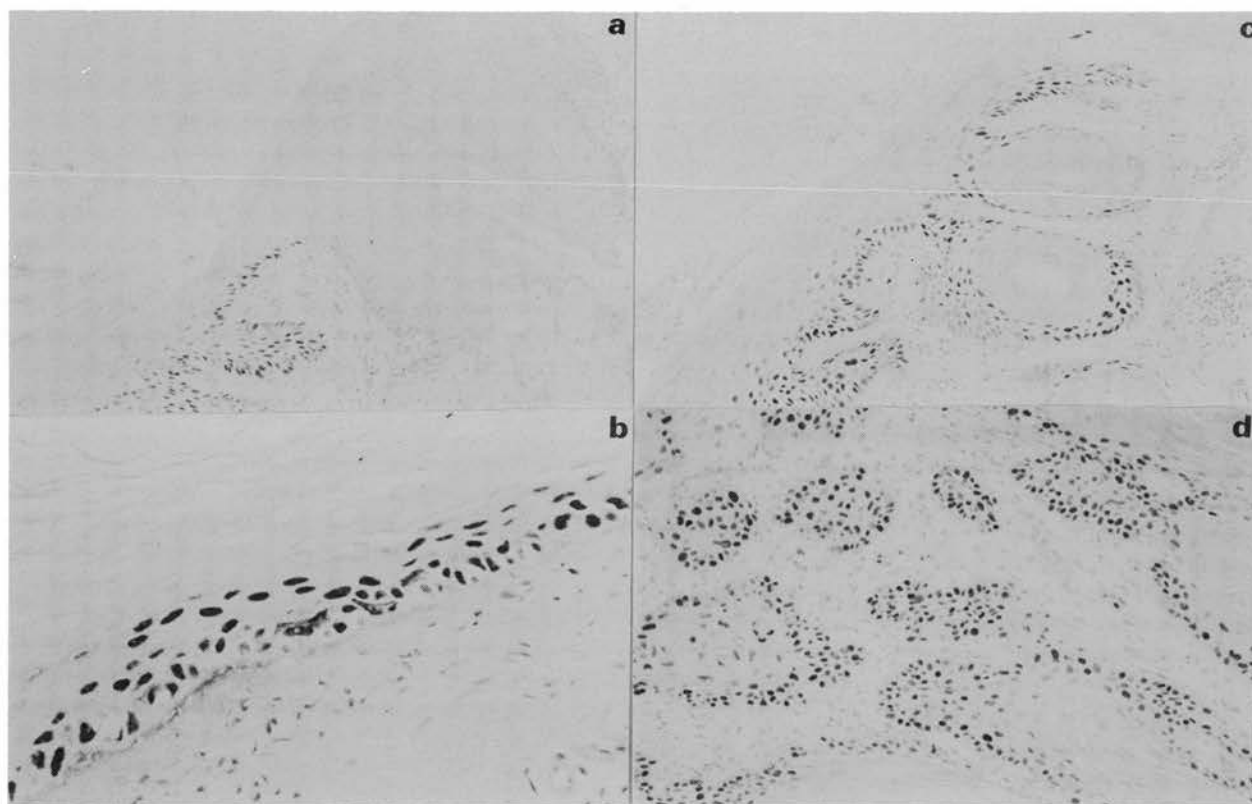
One hundred and twenty-six biopsies from RARs and 75 from ICPs were also screened for the presence of HPV DNA using low-stringency Southern hybridisation with a cocktail of HPV probes, and type-specific PCR for HPV types 1, 2, 5, 8, 6, 11, 16 and 18 (Table II). The details of these results are reported elsewhere (Stark *et al.*, 1994). Overall, no relationship was observed between the presence of accumulated p53 and HPV DNA in premalignant or malignant cutaneous lesions from RARs or ICPs. The prevalences of the specific HPV types 1, 2, 5, 8, 6, 11, 16 and 18 were also too low to determine whether any correlation existed between these HPV types and p53 immunoreactivity.

#### SSCP analysis of p53 immunopositive and immunonegative lesions

SSCP analysis of exons 5–8 of the p53 gene was performed on 28 IECs/SCCs from RARs and ICPs. Fifteen of these were immunopositive (grades 2 and 3) and 13 were immunonegative (including seven with grade 1 staining) (Table III and Figure 3). Overall, SSCP mutations (SSCPs) were detected in 6/28 (21%) malignancies [3/15 (20%) SCCs and 3/13 (23%) IECs]. However, the incidence of mutation was related to the grade of p53 positivity detected by immunocytochemistry with 5/9 (56%) grade 3, 1/6 (17%) grade 2 and no grade 1 lesions showing SSCP. Three of the SSCP were in exon 7 (all grade 3), two in exon 5 (one grade 3, the other grade 2) and one in exon 8 (grade 3). In our series, no SSCP were detected in immunonegative cancers or matched normal skin samples and there was no difference in the number of SSCP present in RARs and ICPs. Direct DNA sequencing of one SCC with a SSCP mutation in exon 7 revealed a C–T transition at codon 248 (Figure 3). SSCP were detected in



**Figure 1** Extent of p53 staining in cutaneous lesions from RARs and ICPs. Grade 1 (hatched) = <10% of cells, grade 2 (cross-hatched) = 10–50% of cells, grade 3 (solid black) = >50% of cells in a lesion showing positive nuclear staining by immunocytochemistry; neg (white) = negative by immunocytochemistry; US, uninvolved, sun-exposed skin; VW, viral wart; K, keratosis; IEC, intraepidermal carcinoma; SCC, squamous cell carcinoma.



**Figure 2** Histological distribution of accumulated p53. **a**, Severely dysplastic keratosis (left) is associated with strong p53 immunostaining as compared with negative normal epidermis (right). **b**, p53 immunostaining is localised to the dysplastic basal cells in actinic keratosis. **c**, Dysplastic basal cells are positive for p53 while the specialised cells in appendages are negative. **d**, Nuclear localisation of p53 in an invasive squamous cell carcinoma from a RAR. p53 immunocytochemistry was performed using PAb Do-7 and a standard ABC/horseradish peroxidase technique.



**Table II** Correlation between presence of HPV DNA and accumulated p53 in cutaneous lesions from RARs and ICPs

Histology	RARs				ICPs			
	HPV+		HPV-		HPV+		HPV-	
	p53+ <sup>a</sup>	p53-	p53+ <sup>a</sup>	p53-	p53+ <sup>a</sup>	p53-	p53+ <sup>a</sup>	p53-
US	0/3	3/3	5/16	11/16	1/1	0/1	2/11	9/11
VWs	1/10	9/10	0/5	5/5	1/5	4/5	1/1	0/1
Ks	4/10	6/10	6/19	13/19	0/3	3/3	3/11	8/11
IECs	8/12	4/12	13/20	7/20	2/4	2/4	13/24	11/24
SCCs	7/15	8/15	10/16	6/16	3/5	2/5	4/10	6/10

<sup>a</sup>Sections with staining in >10% of nuclei in the lesion (grade 2 and 3) were scored as positive. RAR, renal allograft recipient; ICP, immunocompetent patient; US, uninvolved, sun-exposed skin; VW, viral wart; K, keratosis; IEC, intraepidermal carcinoma; SCC, squamous cell carcinoma

**Table III** SSCP analysis of immunopositive and immunonegative tumours from RARs and ICPs

Patients	Histology	No. of ICC positive and negative lesions exhibiting SSCPs			
		Neg	Grade 1	Grade 2	Grade 3
RARs	SCC	0/1	0/3	0/2	3/3
	IEC	0/3	0/1	0/1	1/2
	Normal	0/5	0/2	—	—
ICPs	SCC	—	0/2	0/2	0/2
	IEC	0/2	0/1	1/1	1/2
	Normal	0/5	—	—	—

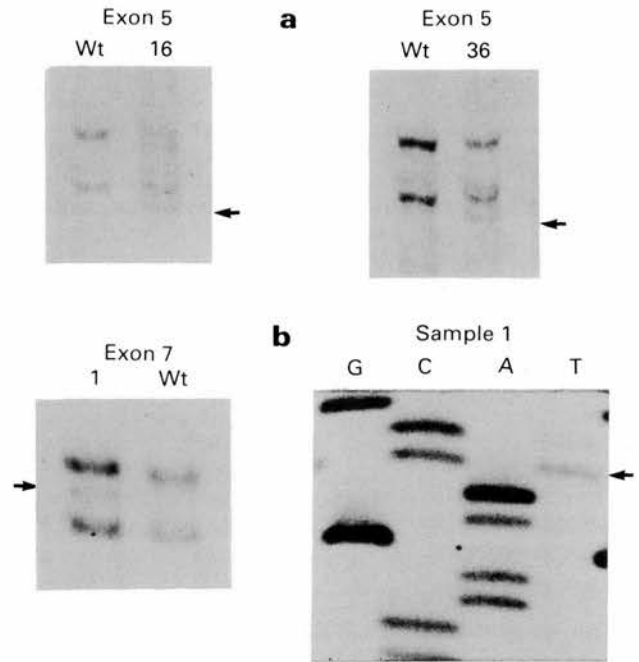
ICC, immunocytochemical; SSCPs, p53 mutations as detected by SSCP analysis; grade 1, <10%, grade 2, 10–50%; grade 3, ≥50% of cells in a lesion showing positive nuclear staining; RAR, renal allograft recipient; ICP, immunocompetent patient, SCC, squamous cell carcinoma; IEC, intraepidermal carcinoma.

three HPV-positive malignancies and one HPV-negative malignancy.

## Discussion

### p53 accumulation and progression in cutaneous carcinogenesis

In this study we have demonstrated the presence of accumulated p53 in over 50% of cutaneous SCCs from both RARs and ICPs suggesting that p53 may play a role in skin carcinogenesis in both populations. This detection level is in broad agreement with previously reported results for SCC in ICPs in which it has ranged from 15% to 56% of lesions (Gusterson *et al.*, 1991; McGregor *et al.*, 1992; Ro *et al.*, 1992). A striking feature of our study was the increase in prevalence and extent of staining which occurred as lesions progressed through the histological spectrum of neoplasia. Indeed, there was a close correlation between the extent of staining in these lesions and the severity of dysplasia. These results strongly suggest that in skin carcinogenesis, in both RARs and ICPs, accumulation of p53 represents an important step in malignant progression. This hypothesis is supported by recent studies of skin carcinogenesis in p53 null mice, in which inactivation of p53 specifically associates with progression of benign papillomas to SCCs (Kemp *et al.*, 1993). It is important to note, however, that the occurrence of p53 immunoreactivity does not always equate with acquisition of the malignant state since in ICPs accumulated p53 can be demonstrated in solar keratoses, of which only a small proportion progress to invasive carcinoma (Marks *et al.*, 1986). Clearly, other genetic events must contribute to the development of invasive skin malignancies. In this context it is also of interest that we found a small number of SCCs showing p53 staining in superficial dysplastic epidermis and adjacent areas of IEC but not in contiguous tongues of invasive SCC. One possible explanation for this may be that gross chromosomal deletions, involving 17p, have occurred in more invasive malignant elements, abolishing all p53 gene expression.



**Figure 3** a, Examples of SSCP mutations in exons 5 and 7 of p53 in cutaneous malignancies from RARs and ICPs. Using SSCP analysis, a single base change, such as a point mutation, is visualised as a band of altered migration (as indicated by arrows) in a polyacrylamide gel. Samples 1 and 16 = squamous cell carcinomas from renal allograft recipients, both with grade 3 staining by immunocytochemistry (ICC); sample 36 = an intraepidermal carcinoma from an immunocompetent patient with grade 2 staining by ICC. b, Direct DNA sequencing of exon 7 from sample 1 showing a C-T transition at codon 248 of p53.

### HPV and p53 in cutaneous lesions from RARs and ICPs

E6 oncoproteins from HPV types 16 and 18 can bind to and induce rapid degradation of wild-type p53 (Scheffner *et al.*, 1990; Werness *et al.*, 1990). From observations in anogenital cancers it has been proposed that p53 inactivation occurs either by complexing of wild-type p53 with such viral oncoproteins or, in the absence of virus, by mutational loss of gene function (Crook *et al.*, 1991, 1992; Scheffner *et al.*, 1991, 1992). This concept, however, remains controversial, and other workers have failed to confirm these suggestions (Busby-Earle *et al.*, 1993; Cooper *et al.*, 1993). We have recently reported the prevalence of HPV in cutaneous lesions from RARs (Stark *et al.*, 1994) and suggested that the mechanism by which HPV contributes to skin carcinogenesis may differ from that proposed for anogenital cancer. The present study confirms our previous findings in that we have failed to demonstrate any relationship between the presence or absence of HPV DNA and accumulated p53 in dysplastic or frankly malignant skin lesions from RARs or ICPs. Moreover, p53 mutations were detected by SSCP analysis in both HPV-positive and -negative malignancies. Recently, it has also been demonstrated that the E6 oncoprotein from skin-associated HPV type 8 does not bind to p53, unlike its HPV 16 or 18 equivalent (Steger & Pfister, 1992). Therefore, if HPV is involved in cutaneous carcinogenesis, it must be presumed to act by a different mechanism from that found in anogenital cancer.

### p53 mutations in cutaneous carcinogenesis

In this study SSCP analysis was used to demonstrate mutations in the p53 gene. Although the precise sensitivity of this technique is presently unknown, a recent study in our laboratory involving human colorectal cancer, in which both SSCP analysis and direct sequencing were performed, indi-

cates that approximately 80% of p53 mutations can be detected by SSCP analysis (Cripps *et al.*, manuscript in preparation). The detection of p53 mutations in 21% of SCCs/IECs in our series is in agreement with previous reports for cutaneous cancer (Pierceall *et al.*, 1991; Ro *et al.*, 1992; Campbell *et al.*, 1993a, b). With one exception, these mutations occurred in exons 5 and 7, in keeping with the suggestion that these exons contain mutational hotspots for most human malignancies (Brash *et al.*, 1991; Hollstein *et al.*, 1991; Pierceall *et al.*, 1991; Campbell *et al.*, 1993a; Levine, 1993). Molecular analysis of p53 mutations has previously suggested that the pattern of nucleotide alterations may be tissue dependent and related to the type of mutagenic agent involved (Harris, 1991; Vogelstein & Kinzler, 1992). For instance, CC to TT double-base changes are almost exclusively associated with UV-induced DNA damage (Brash *et al.*, 1991). It is of interest, therefore, that the SCC in our series that was sequenced was found to contain a C–T transition at the codon 248 mutational hotspot, implicating UV radiation in its genesis.

p53 immunocytochemical detection and gene mutations

Immunocytochemistry has been proposed as a rapid and simple means of identification of p53 gene mutations. In the majority of tumours, good correlation has been observed between the presence of immunocytochemically stable p53 and gene mutations determined by sequencing or other methods (Gannon *et al.*, 1990; Iggo *et al.*, 1990; Bodner *et al.*, 1992). However, it is also recognised that immunocytochemically detectable levels of wild-type p53 may occur

in response to DNA injury, and that some p53 mutations do not result in immunocytochemical demonstration of p53 protein (Bodner *et al.*, 1992; Oliner *et al.*, 1992; Wynford-Thomas, 1992; Hall *et al.*, 1993; Lane, 1993). In this study, SSCP analysis of exons 5–8 detected mutations in 6/15 (40%) immunopositive malignancies, with most mutations occurring in tumours with the largest number of positive cells (grade 3 lesions). This indicates that immunocytochemical detection of p53 does not always signify the presence of p53 gene mutations in skin cancers, particularly where there are relatively few positive cells. While the possibility remains that mutations may have occurred in exons other than 5–8, our own experience and that of others studying other common cancers suggests that this is likely to account for only a small proportion of cases. This implies that additional factors may contribute to the accumulation of p53 during the development of at least some skin cancers. Recently, the product of the *mdm-2* gene, which is overexpressed in osteosarcomas, has been shown to bind to and inactivate p53 (Momand *et al.*, 1992; Oliner *et al.*, 1992). It is possible that similar proteins may be present in transformed epidermal cells, complexing with wild-type p53 and rendering it detectable by immunocytochemical methods. The identity of such proteins and their role in the accumulation of p53 and subsequent development of skin cancer remain to be established.

We would like to thank the Scottish Home and Health Department for funding this research, Robert Morris and John Lauder for technical advice and Jill Bubb and Andrew Wyllie for useful discussions.

References

ALLOUB, M.I., BARR, B.B.B., MCLAREN, K.M., SMITH, I.W., BUNNEY, M.H. & SMART, G.E. (1989). Human papillomavirus and lower genital neoplasia in renal transplant patients. *Obstet. Gynecol.*, **68**, 251–258.
ARENDS, M.J., DONALDSON, Y.K., DUVAL, E., WYLLIE, A.H. & BIRD, C.C. (1991). HPV in full thickness cervical biopsies: high prevalence in CIN 2 and CIN 3 detected by a sensitive PCR assay. *J. Pathol.*, **165**, 301–309.
BAKER, S.J., FEARON, E.R., NIGRO, J.M., HAMILTON, S.R., PREISINGER, A.C., JESSUP, J.M., VAN TUINEN, P., LEDBETTER, D.H., BARKER, D.F., NAKAMURA, Y., WHITE, R. & VOGELSTEIN, B. (1989). Chromosome 17p deletions and p53 gene mutations in colorectal carcinomas. *Science*, **249**, 912–915.
BANKS, L., MATLASHEWSKI, G. & CRAWFORD, L. (1986). Isolation of human-p53-specific monoclonal antibodies and their use in the studies of human p53 expression. *Eur. J. Biochem.*, **159**, 529–534.
BARR, B.B.B., BENTON, E.C., MCLAREN, K.M., BUNNEY, M.H., SMITH, I.W., BLESSING, K. & HUNTER, J.A.A. (1989). Human papilloma virus infection and skin cancer in renal allograft recipients. *Lancet*, **i**, 124–129.
BENTON, E.C., SHAHIDULLAH, H. & HUNTER, J.A.A. (1992). Human papillomavirus in the immunosuppressed. *Papillomavirus Rep.*, **3**, 23–26.
BLESSING, K., MCLAREN, K.M., BENTON, E.C., BARR, B.B., BUNNEY, M.H., SMITH, I.W. & BEVERIDGE, G.W. (1989). Histopathology of skin lesions in renal allograft in recipients – an assessment of viral features and dysplasia. *Histopathology*, **14**, 129–139.
BLOHME, I. & LARKO, O. (1984). Premalignant and malignant skin lesions in renal transplant patients. *Transplantation*, **37**, 165–167.
BODNER, S.M., MINNA, J.D., JENSEN, S.M., D'AMICO, D., CARBONE, D., MITSUDOMI, T., FEDORKO, J., BUCHHAGEN, D.L., NAU, M.M., GAZDAR, A.F. & LINNOILA, R.I. (1992). Expression of mutant p53 proteins in lung cancer correlates with the class of p53 gene mutation. *Oncogene*, **7**, 743–749.
BOYLE, J., MACKIE, R.M., BRIGGS, J.D., JUNOR, B.J.R. & AITCHISON, T.C. (1984). Cancer, warts and sunshine in renal transplant patients. A case control study. *Lancet*, **i**, 702–705.
BRASH, D.E., RUDOLPH, J.A., SIMON, J.A., LIN, A., MCKENNA, G.J., BADEN, H.P., HALPERIN, A.J. & PONTEN, J. (1991). A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc. Natl Acad. Sci. USA*, **88**, 10124–10128.
BURNS, J.E., BAIRD, L.J., CLARK, P.A., BURNS, K., EDINGTON, K., CHAPMAN, C., MITCHELL, C.R., ROBERTSON, G., SOUTAR, D. & PARKINSON, E.K. (1993). Gene mutations and increased levels of p53 protein in human squamous cell carcinomas and their cell lines. *Br. J. Cancer*, **67**, 1274–1284.
BUSBY-EARLE, R.M.C., STEEL, C.M. & BIRD, C.C. (1993). Cervical carcinoma: low frequency of allele loss at loci implicated in other common malignancies. *Br. J. Cancer*, **67**, 71–75.
CAMPBELL, C., QUINN, A.G., RO, Y.-S., ANGUS, B. & REES, J.L. (1993a). p53 mutations are a common and early event which precede tumour invasion in squamous cell neoplasia of the skin. *J. Invest. Dermatol.*, **100**, 746–748.
CAMPBELL, C., QUINN, A.G., ANGUS, B. & REES, J.L. (1993b). The relation between p53 mutation and p53 immunostaining in non-melanoma skin cancer. *Br. J. Dermatol.*, **129**, 235–241.
CLARKE, A.R., PURDIE, C.A., HARRISON, D.J., MORRIS, R.G., BIRD, C.C., HOOPER, M.L. & WYLLIE, A.H. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, **362**, 849–852.
COOPER, K., HERRINGTON, C.S., EVANS, M.F., GATTER, K.C. & MCGEE, J.O.D. (1993). p53 antigen in cervical condylomata, intraepithelial neoplasia and carcinoma: relationship to HPV infection and integration. *J. Pathol.*, **171**, 27–34.
CROOK, T., WREDE, D., TIDY, J.A., SCHOLFIELD, J., CRAWFORD, L. & VOUSDEN, K.H. (1991). Status of c-myc, p53 and retinoblastoma genes in human papillomavirus positive and negative squamous cell carcinomas of the anus. *Oncogene*, **6**, 1251–1257.
CROOK, T., WREDE, D., TIDY, J.A., MASON, W.P., EVANS, D.J. & VOUSDEN, K.H. (1992). Clonal p53 mutation in primary cervical cancer: association with human papillomavirus-negative tumours. *Lancet*, **339**, 1070–1073.
DEBBAS, M. & WHITE, E. (1993). Wild type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev.*, **7**, 546–554.
DONEHOWER, L.A., HARVEY, M., SLAGLE, B.L., MCARTHUR, M.J., MONTGOMERY, C.A., BUTEL, J.S. & BRADLEY, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, **356**, 215–221.
GANNON, J.V., GREAVES, R., IGGO, R. & LANE, D.P. (1990). Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J.*, **9**, 1595–1602.



- GUSTERSON, B.A., ANBAZHAGAN, R., WARREN, W., MIDGLEY, C., LANE, D.P., O'HARE, M., STAMPS, A., CARTER, R. & JAYATILAKE, H. (1991). Expression of p53 in premalignant and malignant squamous epithelium. *Oncogene*, **6**, 1785-1789.
- HALL, P.A., MCKEE, P.H., DU, P., MANAGE, H., DOVER, R. & LANE, D.P. (1993). High levels of p53 protein in UV-irradiated normal human skin. *Oncogene*, **8**, 203-207.
- HARRIS, A.L. (1991). Telling changes of base. *Nature*, **350**, 377-378.
- HARTWELL, L. (1992). Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell*, **71**, 543-546.
- HOLGATE, C.S., JACKSON, P., POLLARD, K., LUNNY, D. & BIRD, C.C. (1991). Effect of fixation on T and B lymphocyte surface membrane antigen demonstration in paraffin processed tissue. *J. Pathol.*, **149**, 293-300.
- HOLLSTEIN, M., SIDRANSKY, D., VOGELSTEIN, B. & HARRIS, C.C. (1991). P53 mutations in human cancers. *Science*, **253**, 49-53.
- IGGO, R., GATTER, K., BARTEK, J., LANE, D.P. & HARRIS, A.L. (1990). Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet*, **335**, 675-679.
- KASTAN, M.B., ONYEKWERE, O., SIDRANSKY, D., VOGELSTEIN, B. & CRAIG, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, **51**, 6304-6311.
- KEMP, C.J., DONEHOWER, L.A., BRADLEY, A. & BALMAIN, A. (1993). Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically-induced skin tumours. *Cell*, **74**, 813-822.
- LANE, D.P. (1992). p53, guardian of the genome. *Nature*, **358**, 15-16.
- LANE, D.P. (1993). A death in the life of p53. *Nature*, **362**, 786-787.
- LEVINE, A.J. (1993). The p53 tumour suppressor gene and product, 11th Ernst Klenk Lecture. *Biol. Chem. Hoppe-Seyler*, **374**, 227-233.
- MARKS, R., FOLEY, P., GOODMAN, G., HAGE, B.H. & SELWOOD, T.S. (1986). Spontaneous remission of solar keratoses - the case for conservative management. *Br. J. Dermatol.*, **115**, 649-655.
- MCGREGOR, J.M., YU, C.C.-W., DUBLIN, E.A., LEVISON, D.A. & MACDONALD, D.M. (1992). Aberrant expression of p53 tumour-suppressor gene in non-melanoma skin cancer. *Br. J. Dermatol.*, **127**, 463-469.
- MILNER, J. & MEDCALF, E.A. (1991). Cotranslation of activated mutant p53 with wild type drives the wild type P53 protein into a mutant conformation. *Cell*, **65**, 774-785.
- MILNER, J. & COOK, A. (1986). Visualisation, by immunocytochemistry, of p53 at the plasma membrane of both non-transformed and SV40-transformed cells. *EMBO J.*, **9**, 2885-2889.
- MOMAND, J., ZAMBETTI, G.P., OLSON, D.C., GEORGES, D.L. & LEVINE, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53 mediated transactivation. *Cell*, **69**, 1237-1245.
- MONTENARH, M. (1992). Biochemical properties of the growth suppressor oncoprotein p53. *Oncogene*, **7**, 1673-1680.
- MORAN, E. (1993). Interaction of adenoviral proteins with pRB and p53. *FASEB J.*, **7**, 880-885.
- NIGRO, J.M., BAKER, S.J., PREISINGER, A.C., JESSUP, J.M., HOSTETTER, R., CLEARY, K., BIGNER, S.H., DAVIDSON, N., BAYLIN, S., DEVILEE, P., GLOVER, T., COLLINS, F.S., WESTON, A., MODALI, R., HARRIS, C.C. & VOGELSTEIN, B. (1989). Mutations in the p53 gene occur in diverse human tumour types. *Nature*, **342**, 705-708.
- OLINER, J.D., KINZLER, K.W., MEITZER, P.S., GEORGES, D.L. & VOGELSTEIN, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature*, **358**, 80-83.
- PIERCEALL, W.E., MUKHOPADHYAY, T., GOLDBERG, L.H. & ANANTHASWAMY, H.N. (1991). Mutations in the p53 tumour suppressor gene in human cutaneous squamous cell carcinomas. *Mol. Carcinogen*, **4**, 445-449.
- PURDIE, C.A., O'GRADY, J., PIRIS, J., WYLLIE, A.H. & BIRD, C.C. (1991). p53 expression in colorectal tumours. *Am. J. Pathol.*, **138**, 807-813.
- PURDIE, C.A., HARRISON, D.J., PETER, A., DOBBIE, L., WHITE, S., HOWIE, S.E.M., SALTER, D.M., BIRD, C.C., WYLLIE, A.H., HOOPER, M.L. & CLARKE, A.R. (1994). Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. (in press).
- RO, Y.-S., VOJTESEK, B., COOPER, P.N., LEE, J.A., HARRISON, D., ANGUS, B., REES, J., HORNE, C.H.W. & LANE, D.P. (1992). p53 protein expression in benign and malignant squamous and melanocytic skin tumours - an immunohistochemical study. *J. Invest. Dermatol.*, **98**, 540-544.
- RUDLINGER, R., SMITH, I.W., BUNNEY, M.H. & HUNTER, J.A.A. (1986). Human papillomavirus infections in a group of renal transplant recipients. *Br. J. Dermatol.*, **115**, 681-692.
- SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. (1989). *Molecular Cloning. A Laboratory Manual*, 2nd edn, pp. E3-E4. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.
- SCHEFFNER, M., WERNESS, B.A., HULBREGTSE, J.M., LEVINE, A.J. & HOWLEY, P.M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*, **63**, 1129-1136.
- SCHEFFNER, M., MUNGER, K., BYRNE, J.C. & HOWLEY, P.M. (1991). The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc. Natl Acad. Sci. USA*, **88**, 5523-5527.
- SCHEFFNER, M., TAKAHASHI, T., HULBREGTSE, J.M., MINNA, J.D. & HOWLEY, P.M. (1992). Interaction of the human papillomavirus type 16 E6 oncoprotein with wild-type and mutant human p53 proteins. *J. Virol*, **66**, 5100-5105.
- SHUTTLEWORTH, D., MARKS, R., GRIFFIN, P.J.A. & SALAMAN, J.R. (1987). Dysplastic epidermal change in immunosuppressed patients with renal transplants. *Q.J. Med.*, **243**, 609-616.
- SRIVASTAVA, S., ZOU, Z., PIROLLO, K., BLATTNER, W. & CHANG, E.H. (1990). Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature*, **348**, 747-749.
- STARK, L.A., ARENDS, M.J., MCLAREN, K.M., BENTON, E.C., SHAHIDULLAH, H., HUNTER, J.A.A. & BIRD, C.C. (1994). Prevalence of human papillomavirus DNA in cutaneous neoplasms from renal allograft recipients supports a possible viral role in tumour promotion. *Br. J. Cancer*, **69**, 222-229.
- STEGER, G. & PFISTER, H. (1992). *in vitro* expressed HPV 8 E6 protein does not bind p53. *Arch. Dermatol.*, **125**, 355-360.
- STREILEIN, J.W. (1991). Immunogenetic factors in skin cancer. *New Engl J. Med.*, **325**, 885-886.
- UNGER, T., NAU, M.N., SEGAL, S. & MINNA, J.D. (1992). p53: a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. *EMBO J.*, **11**, 1383-1390.
- VOGELSTEIN, B. & KINZLER, K.W. (1992). p53 function and dysfunction. *Cell*, **70**, 523-526.
- VOJTESEK, B., BARTEK, J., MIDGLEY, C.A. & LANE, D.P. (1992). An immunochemical analysis of the human nuclear phosphoprotein p53 new monoclonal antibodies and epitope mapping using recombinant p53. *J. Immunol. Methods*, **151**, 237-244.
- WERNESS, B.A., LEVINE, A.J. & HOWLEY, P.M. (1990). Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science*, **248**, 76-79.
- WYNFORD-THOMAS, D. (1992). p53 in tumour pathology: can we trust immunocytochemistry? *J. Pathol.*, **166**, 329-330.
- YEW, P.R. & BERK, A.J. (1992). Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature*, **357**, 82-85.